Quantitative analysis of SMN gene copies in spinal muscular atrophy

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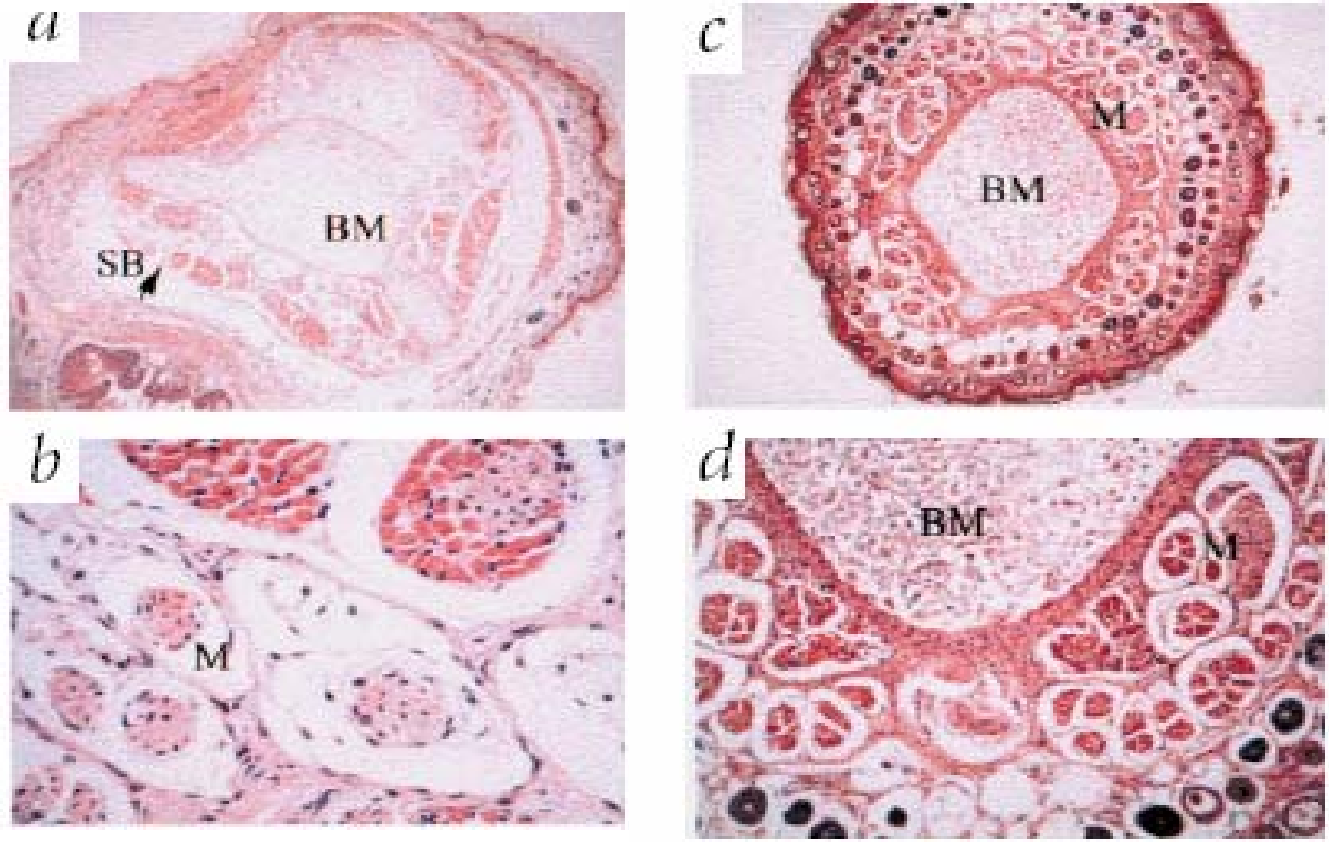
2006.01.18

脊髓肌肉萎縮症

- 第一型(Werdnig-Hoffman disease)，一般在六個月前發病。
- 第二型又稱中間型(Dubowitz disease)， 一般在一歲半前發病。
- 第三型又稱爲庫格勃-韋蘭德症(Kugelberg-Welander disease)， 約在一歲半後發病

疾病的確定診斷

- 血中肌肉酵素的高低
- 肌電圖
- 肌肉切片
- 基因檢查



The type 2 SMA-like mouse tail(a,b)
Normal control(c,d)

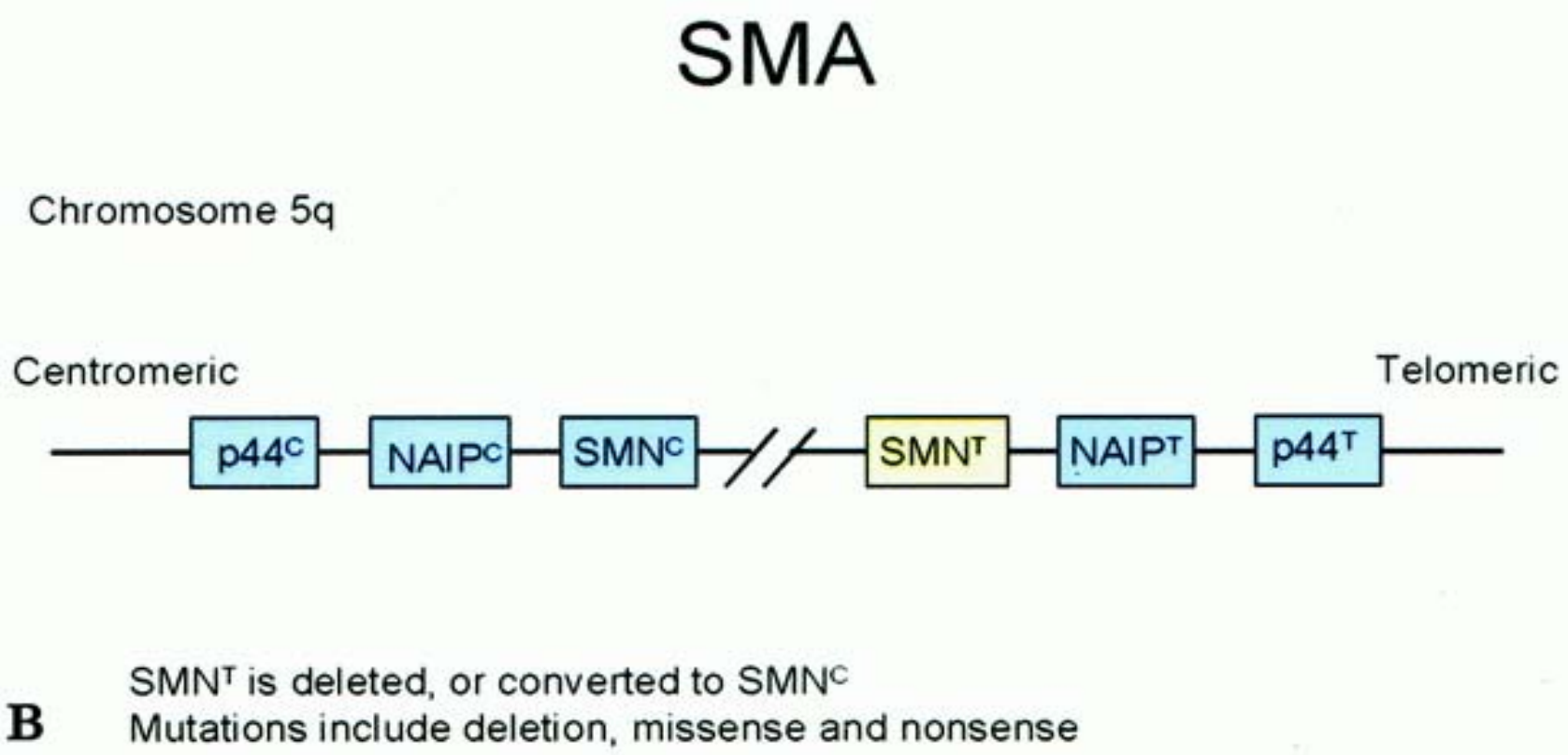


Figure. Genetic mechanisms in three motor neuron diseases. (B) Spinal muscular atrophy (SMA). In SMA, there are two copies of the *SMN* (survival motor neuron), *NAIP* (neuronal apoptosis inhibitory polypeptide), and *p44c* genes. In this condition, the telomeric copy of *SMN*, SMN^T, is deleted or converted to SMN^C. The *NAIP* and *p44c* genes may also be mutant.
From: Orrell: Neurology, Volume 57(1).July 10, 2001.9-17

Table 2 Chromosomes in the general population

Chromosomes with	No. in general population	Frequency
No *SMN1*	11	1.5%
1 *SMN1*	685	91.3%
2 *SMN1*	54	7.2%
Total	750	100%

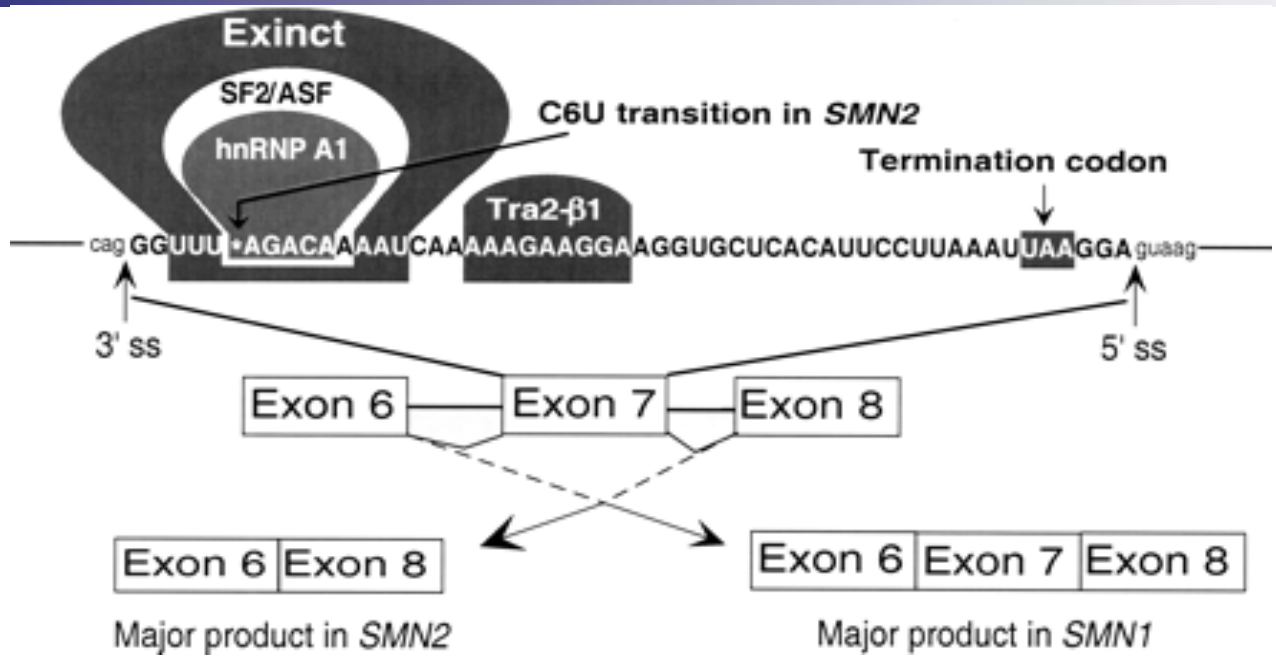


FIGURE 1. Schematic diagram of *SMN* construct containing C6U mutation within exon 7 and the preferred splicing pathways. Tra2-ESE is located in the middle of exon 7 (Hofmann et al. 2000), whereas SF2/ASF-ESE is located toward the 3' ss of exon 7 (Cartegni and Krainer 2002) and overlaps with hnRNP A1-ESS (Kashima and Manley 2003). Earlier reports suggest that C6U (indicated by a star) abrogates SF2/ASF-ESE and/or creates hnRNP A1-ESS, producing exon 7-skipped product in *SMN2*. We have recently shown that C6U creates an extended inhibitory context (Exinct) that causes exon 7 skipping (Singh et al. 2004). (ss) Splice site.

SMN1/SMN2

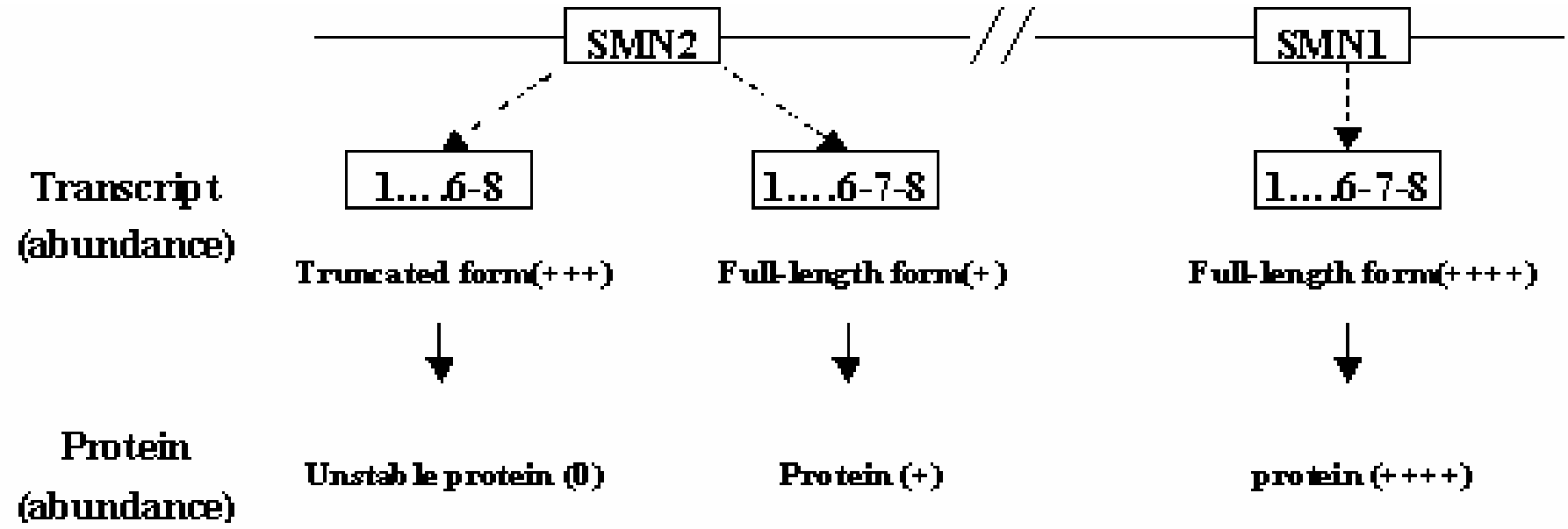
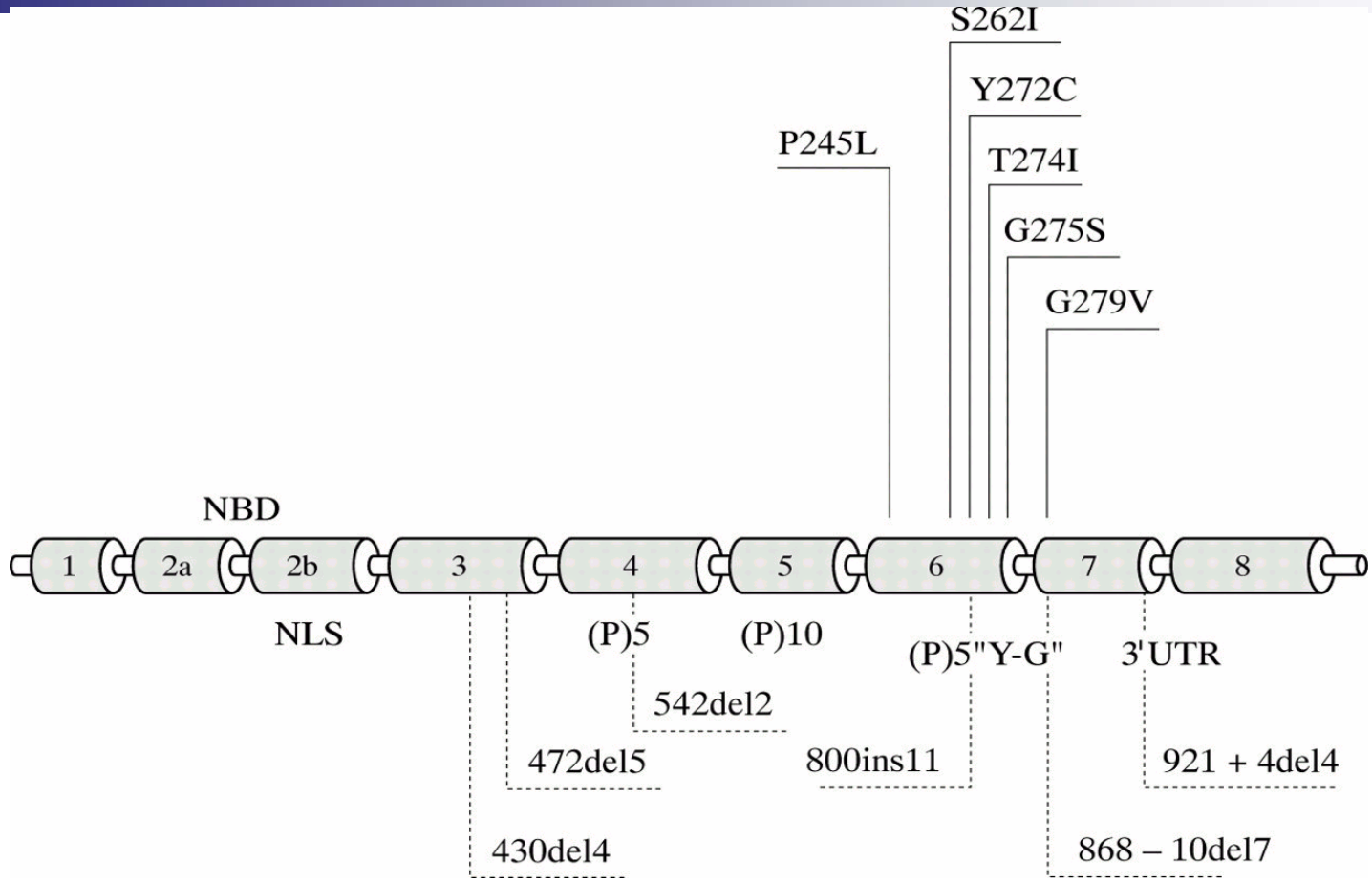


Figure 2. Genomic organization of the SMA locus in human healthy individual.

SMN1/SMN2

	Exon 6	Exon 7-6	Intron 7-362	Intron 7-477	Exon 8
SMN1	G	C	A	A	G
SMN2	A	T	G	G	A



The topographic distribution of the SMN protein domains encoded by the SMN gene and various microrearrangements and missense mutations identified in the SMN gene. NBD=nucleic acid binding domain, NLS=nuclear localisation signal, (P)5=stretch of five prolines, (P)10=stretch of 10 prolines, "Y-G"="Y-G box".

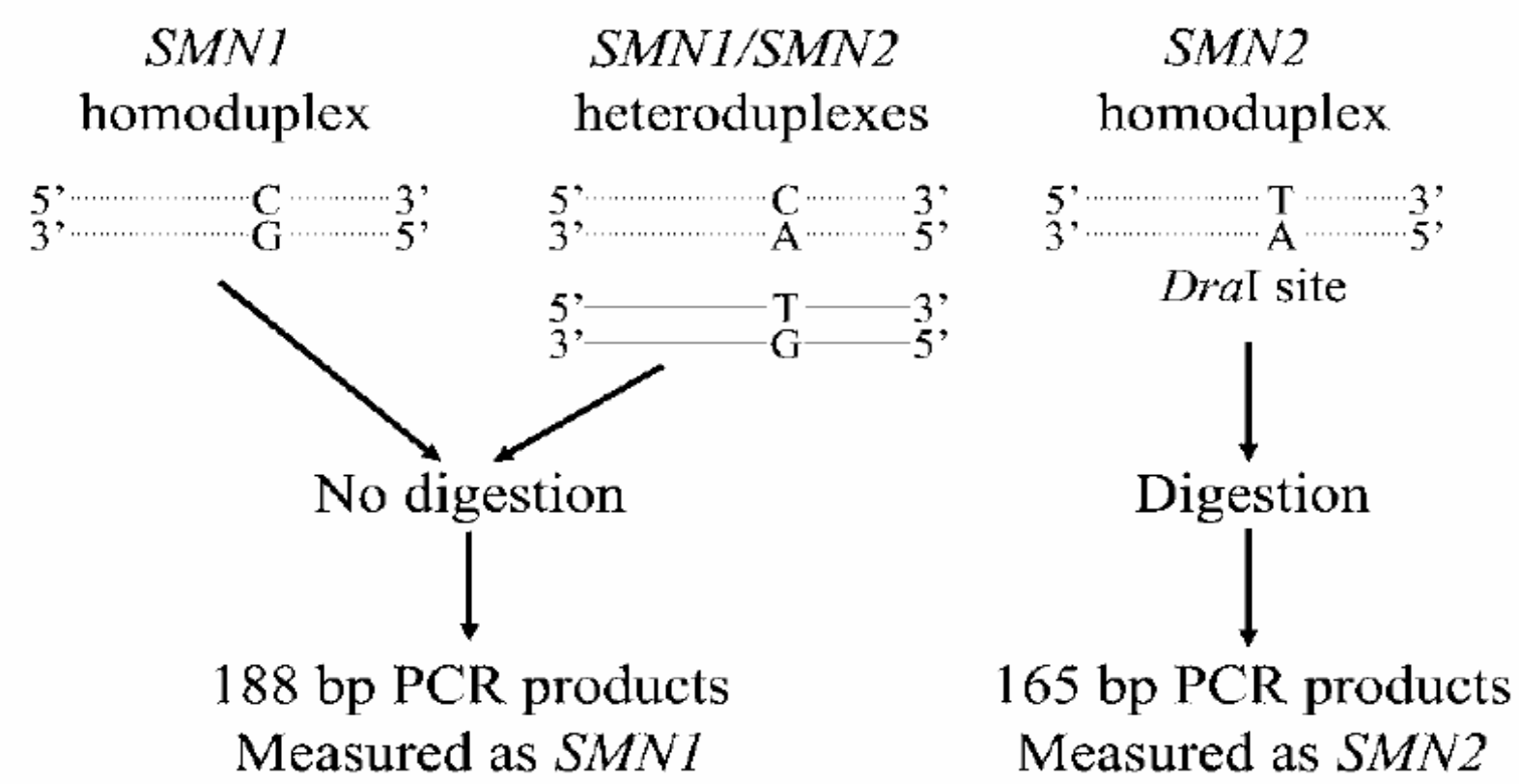
Mutations involved in SMA

Table 1 Mutations involved in SMA

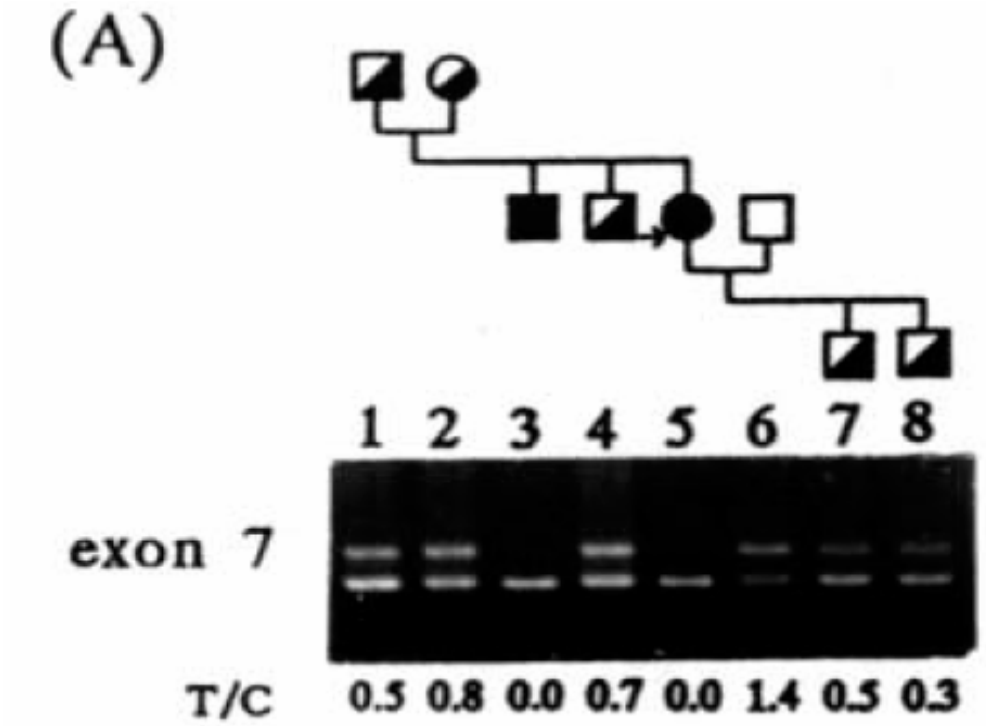
No	*Gene*	*Type of mutation*	*Reference*
93%	SMN^T	Homozygous deletion of exons 7 & 8	17
5.6%	SMN^T	Homozygous deletion of exon 7 only	17
1	SMN^T	Missense mutation on codon 245 (P245L)	35
1	SMN^T	Missense mutation on codon 262 (S262I)	34
1	SMN^T	Missense mutation on codon 272 (Y272C)	17
1	SMN^T	Missense mutation on codon 274 (T274I)	34
1	SMN^T	Missense mutation on codon 275 (G275S)	32
1	SMN^T	Missense mutation on codon 279 (G279V)	33
1	SMN^T	4 bp microdeletion in 5' splice donor site of intron 7 (921+4del4)	17
1	SMN^T	7 bp microdeletion in 3' splice acceptor site of exon 6 (868−10del7)	17
4	SMN^T	4 bp microdeletion on exon 3 (430del4)	36
1	SMN^T	Compound heterozygote 1st copy - 5 bp microdeletion on exon 3 (472del5) 2nd copy - deletion	37
1	SMN^T	Compound heterozygote 1st copy - 2 bp microdeletion on exon 2 (542del2) 2nd copy - deletion or conversion	38
1	SMN^T	11 bp duplication on exon 6 (800ins11)	39
45% of SMA I and 18% of SMA I,II,III	NAIP	Homozygous deletion	18
73% of SMA I	$p44^T$	Homozygous deletion	20
15% of SMA I,II,III	$p44^T$	Homozygous deletion	19

J Med Genet 1999;**36**:1–8

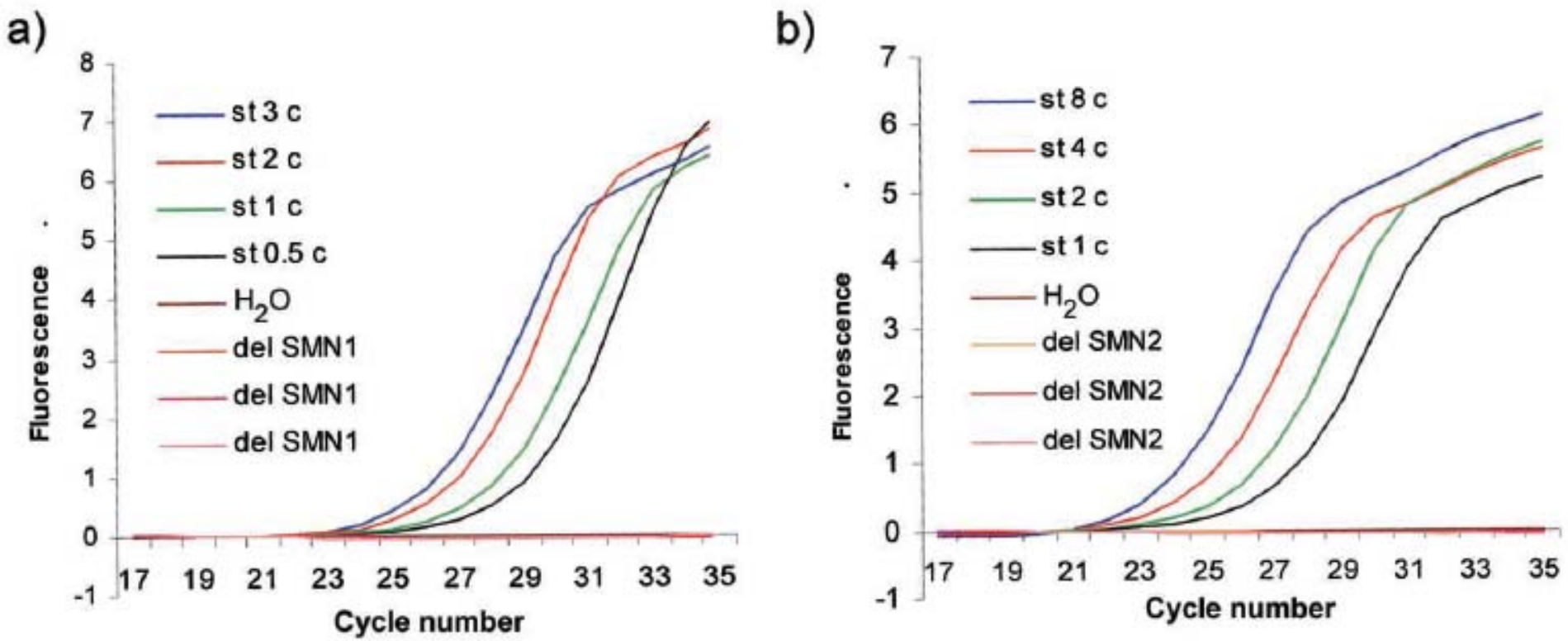
The PCR products of exons 7 of the SMN gene digested with restriction enzyme *Dra*I



The PCR products of exons 7 of the SMN gene digested with restriction enzyme *Dra*I



Quantitative Real-Time PCR of SMN1 and SMN2 Copy Numbers



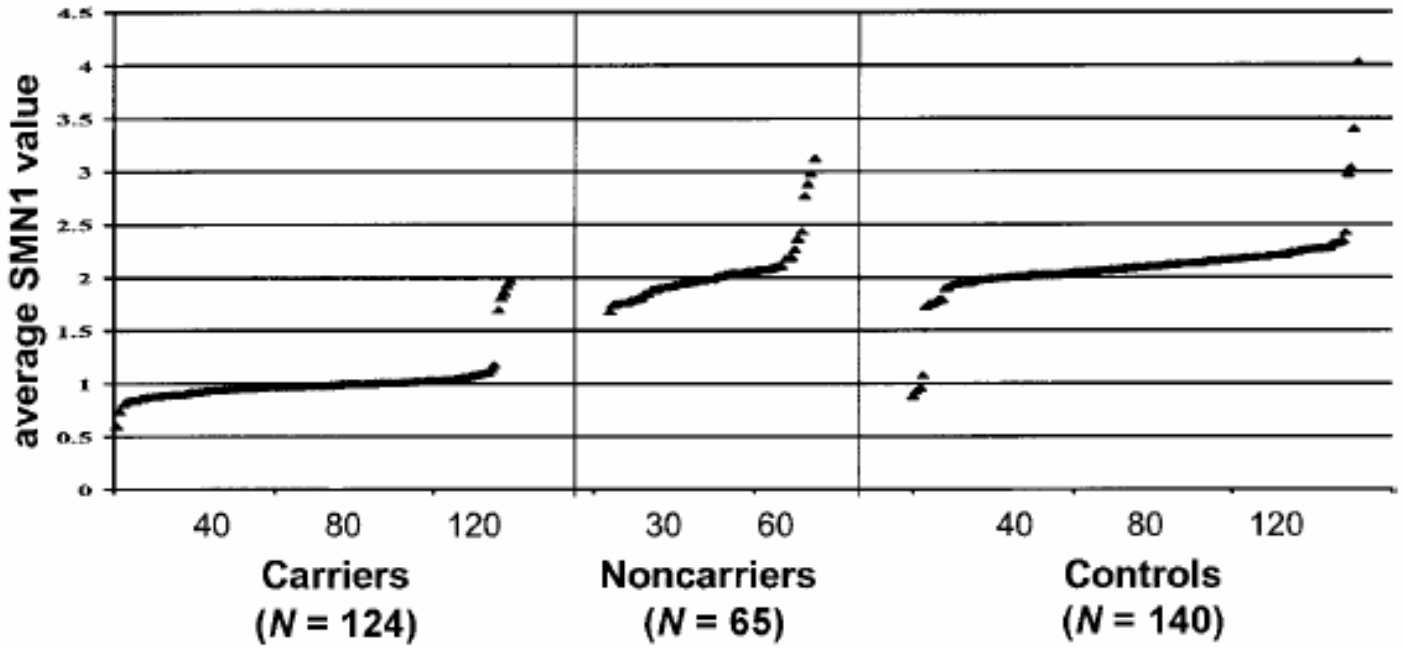


Figure 2 Plot of the average SMN1 values for carriers ($N = 124$), noncarriers (unaffected sibs of patients with SMA) ($N = 65$), and controls ($N = 140$).

M Feldkotter et al. Am J Hum Genet 2002;70:358-368	1 copy SMN2	2 copy SMN2	3 copy SMN2	4 copy SMN2	Total
Type I	13(6.9%)	138(73.4%)	37(19.7%)	0	188
Type II	0	12(10.9%)	90(81.8%)	8(7.3%)	110
Type III	0	3(3.9%)	39(50.6%)	35(45.5%)	77

V Chan et al. J Neurol 2004;51: 1089-1093	1 copy SMN2	2 copy SMN2	3 copy SMN2	4 copy SMN2	Total
Type I	0	2	0	0	2
Type II	0	3	7	0	10
Type III	0	5	5	3*	13

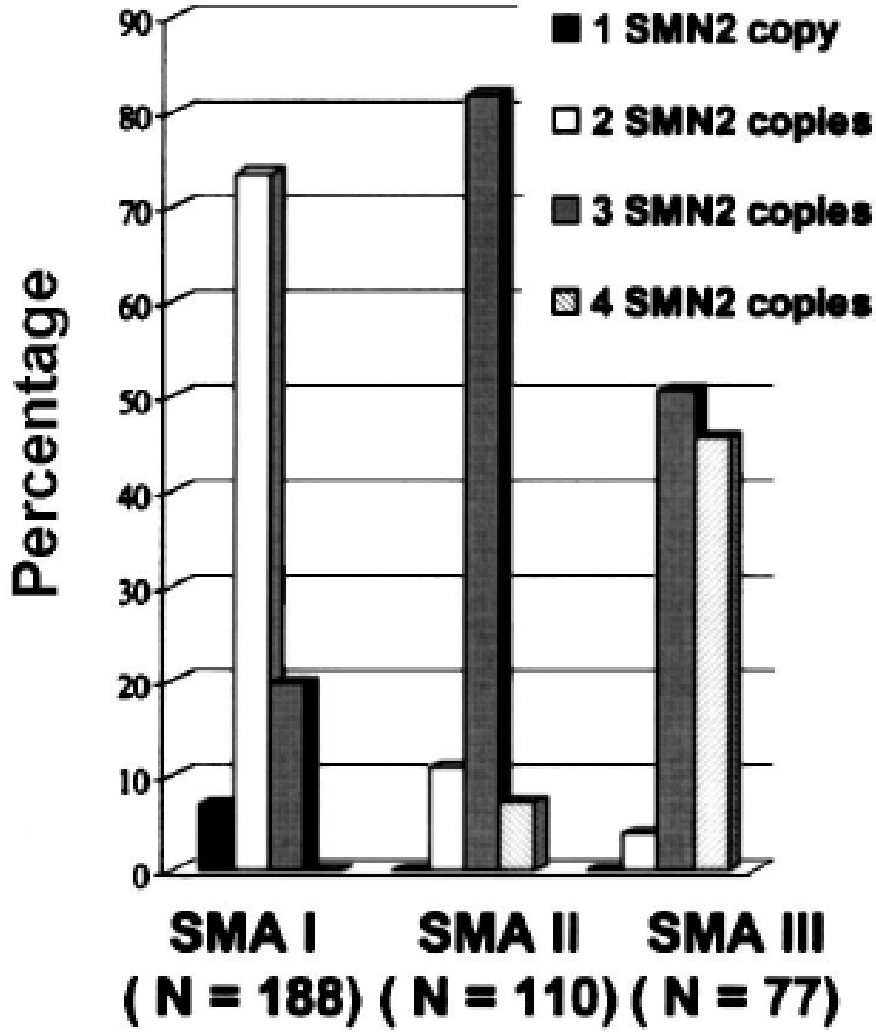


Figure 3 Diagram of the frequency of patients with type I, type II, and type III SMA versus SMN2 copy number.

M Feldkotter et al. Am J Hum Genet 2002;70:358-368	1 copy SMN1	2 copy SMN1	3 copy SMN1	4 copy SMN1	Total
Carrier	119(96.0%)	5(4.0%)	0	0	124
Noncarrier	0	61(93.8%)	4(6.2%)	0	65
Controls	4(2.9%)	132(94.3%)	3(2.1%)	1(0.7%)	140

HUMAN MUTATION 25:460–467 (2005)

METHODS

Quantitative Analysis of *SMN1* and *SMN2* Genes Based on DHPLC: A Highly Efficient and Reliable Carrier-Screening Test

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Protocol

DNA extraction

↓

PCR

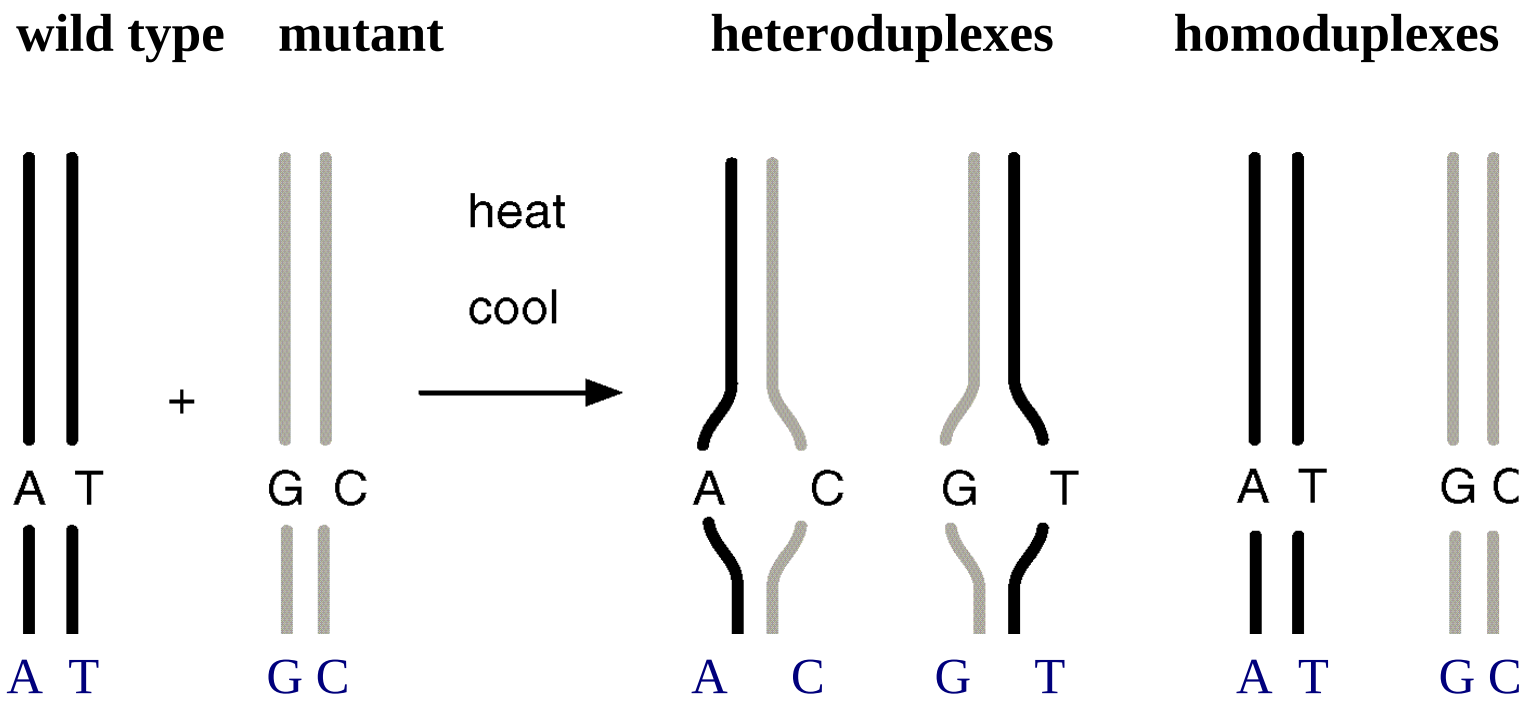
↓

DHPLC

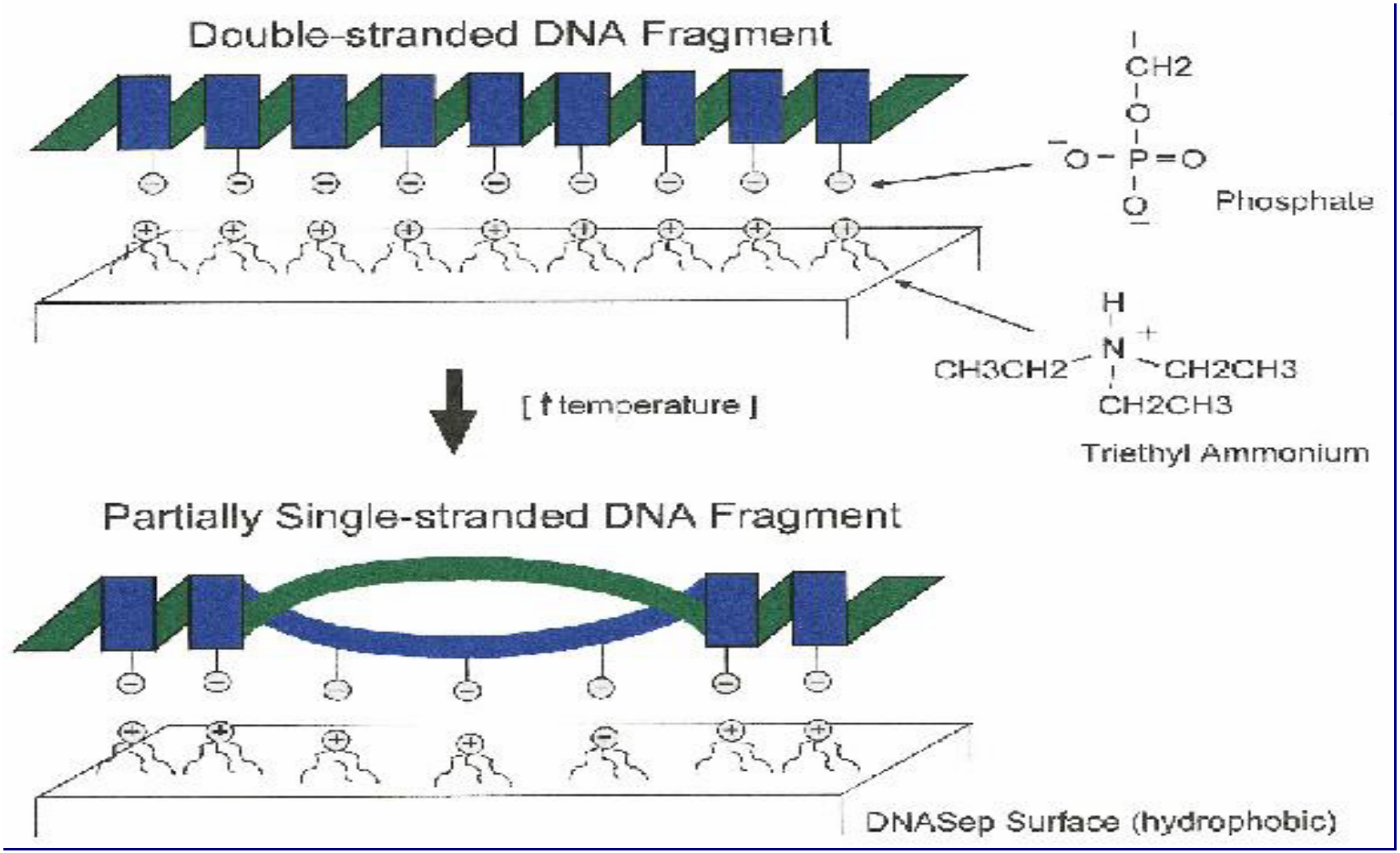
↓

DATA analysis

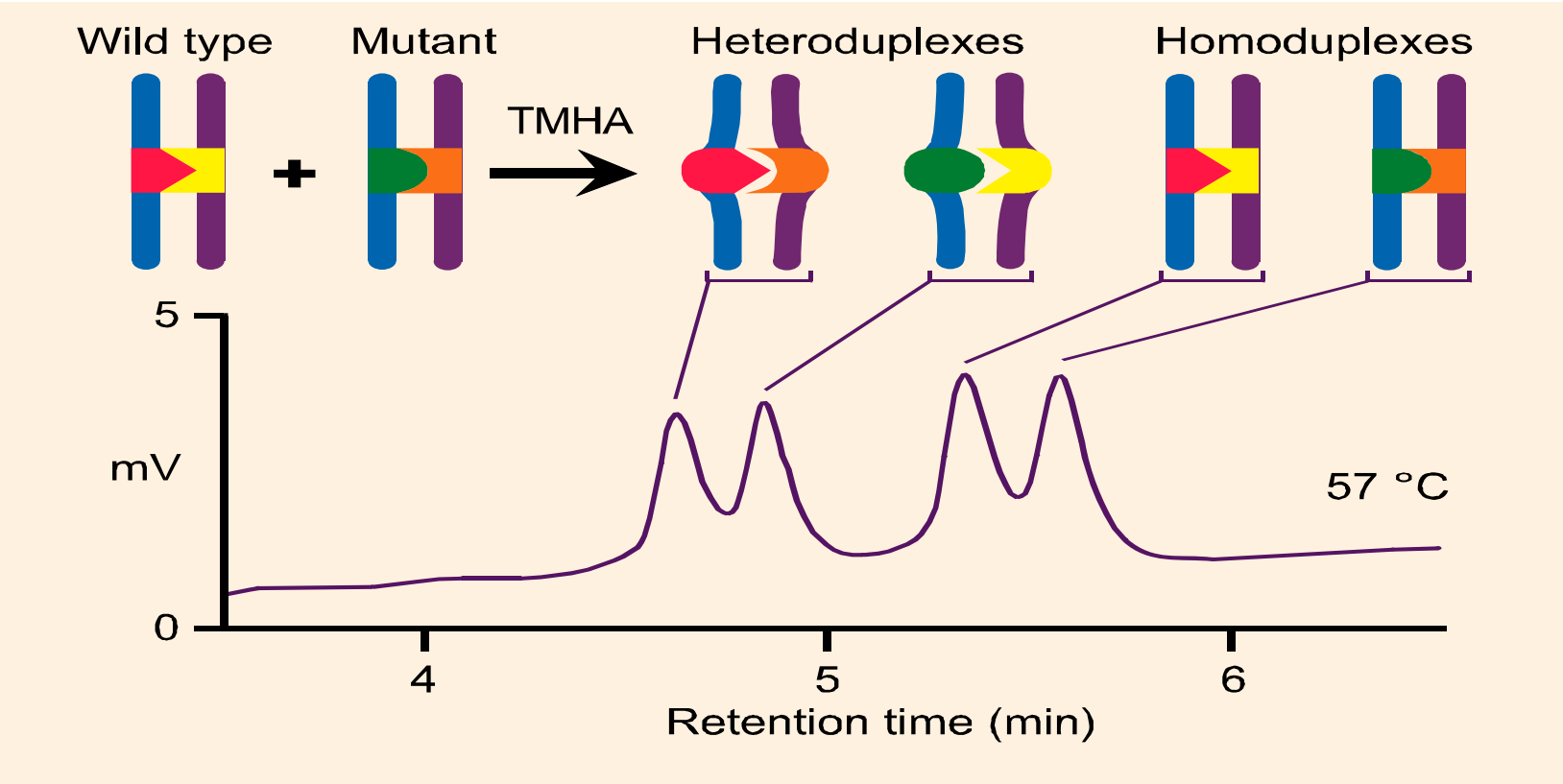
Heteroduplex Analysis



DHPLC


Double-stranded DNA Fragment
CH2
O
O
P=O
O
Phosphate
H
N
CH3CH2
CH2CH3
CH2CH3
Triethyl Ammonium
[↑ temperature]
Partially Single-stranded DNA Fragment
DNASep Surface (hydrophobic)

Examples of Partial Denaturing Operation for Mutation Detection



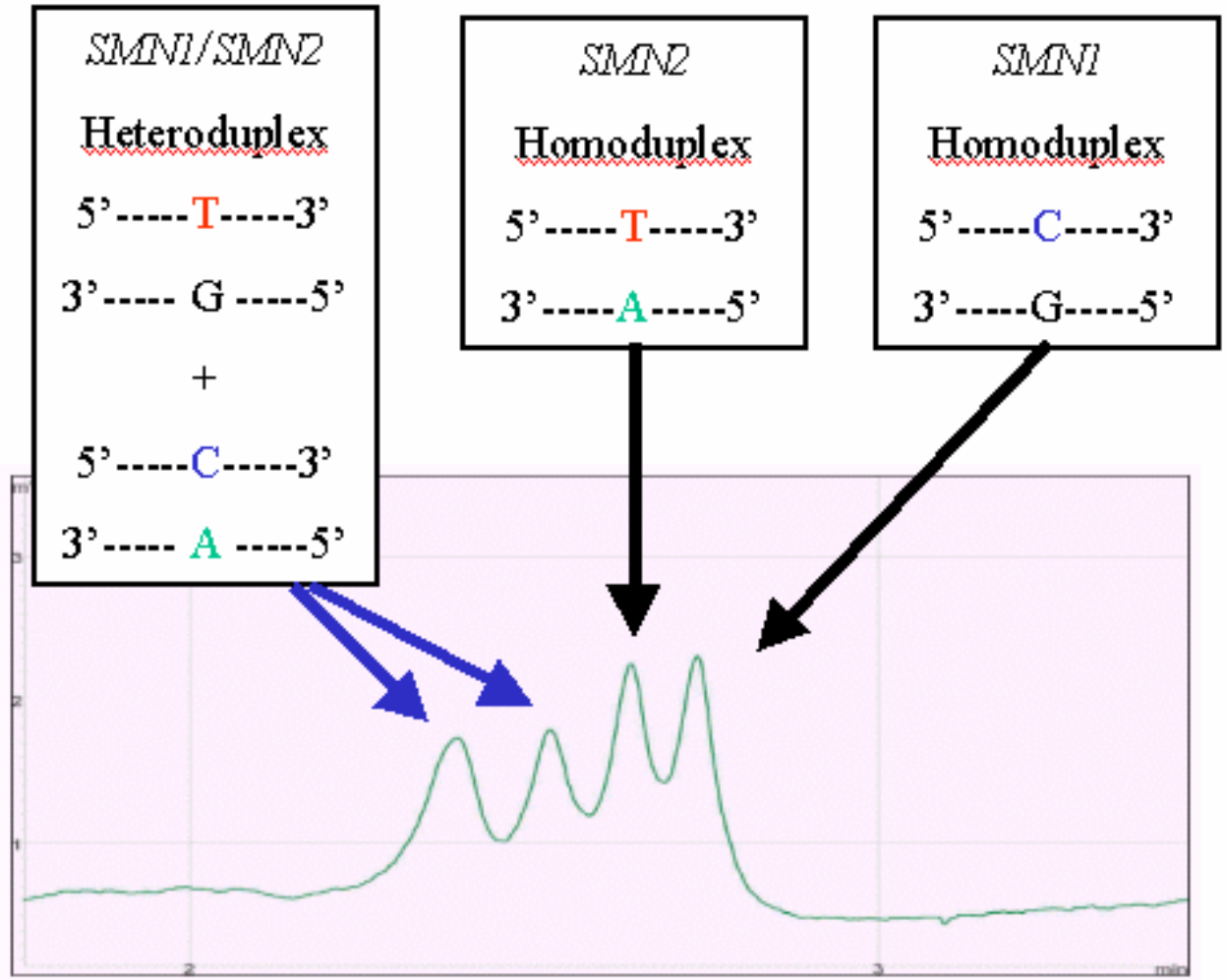
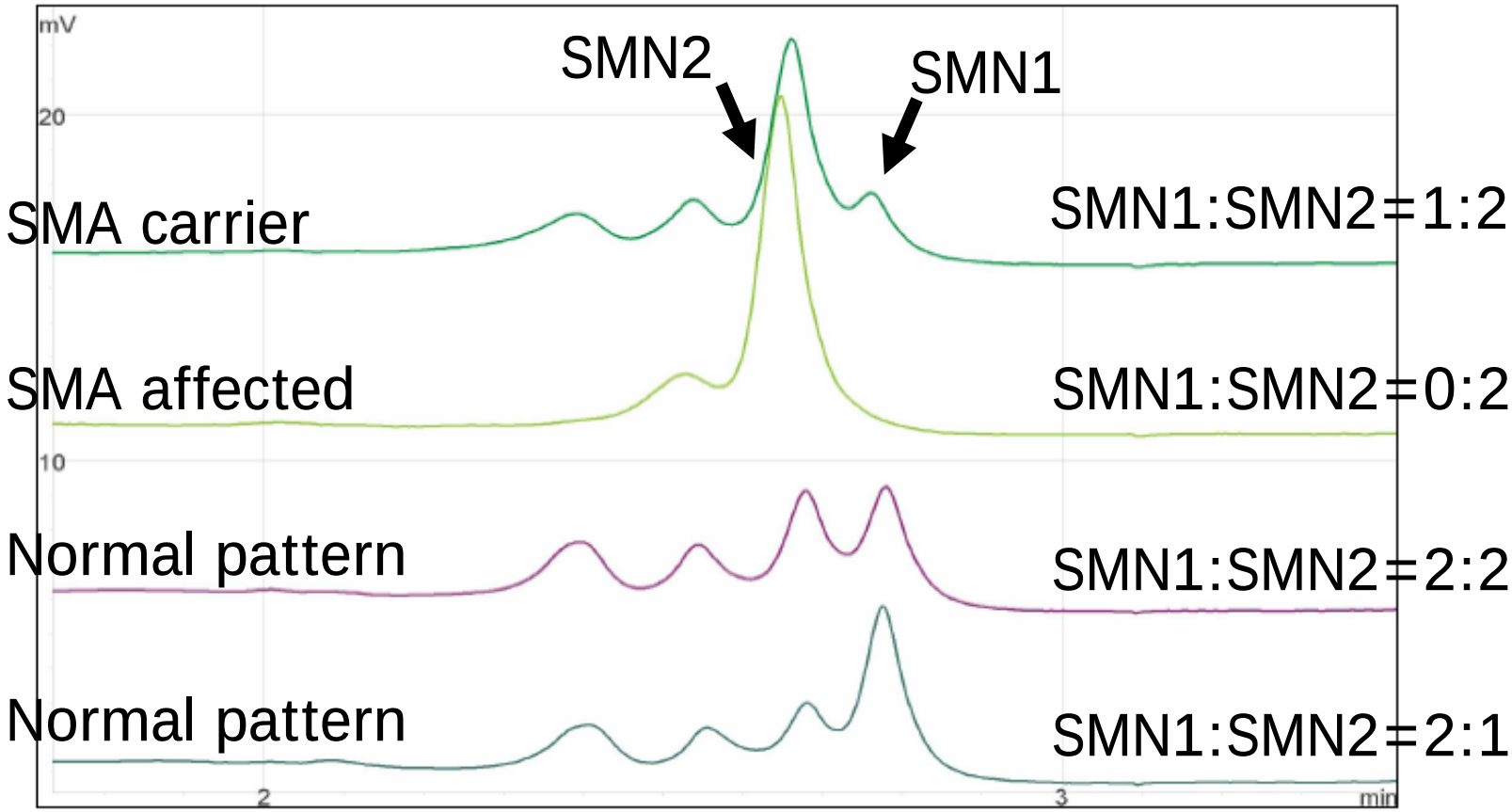


FIG. 1.DHPLC of an individual with an *SMN1/SMN2* gene(wild type)

Chromatography of DHPLC



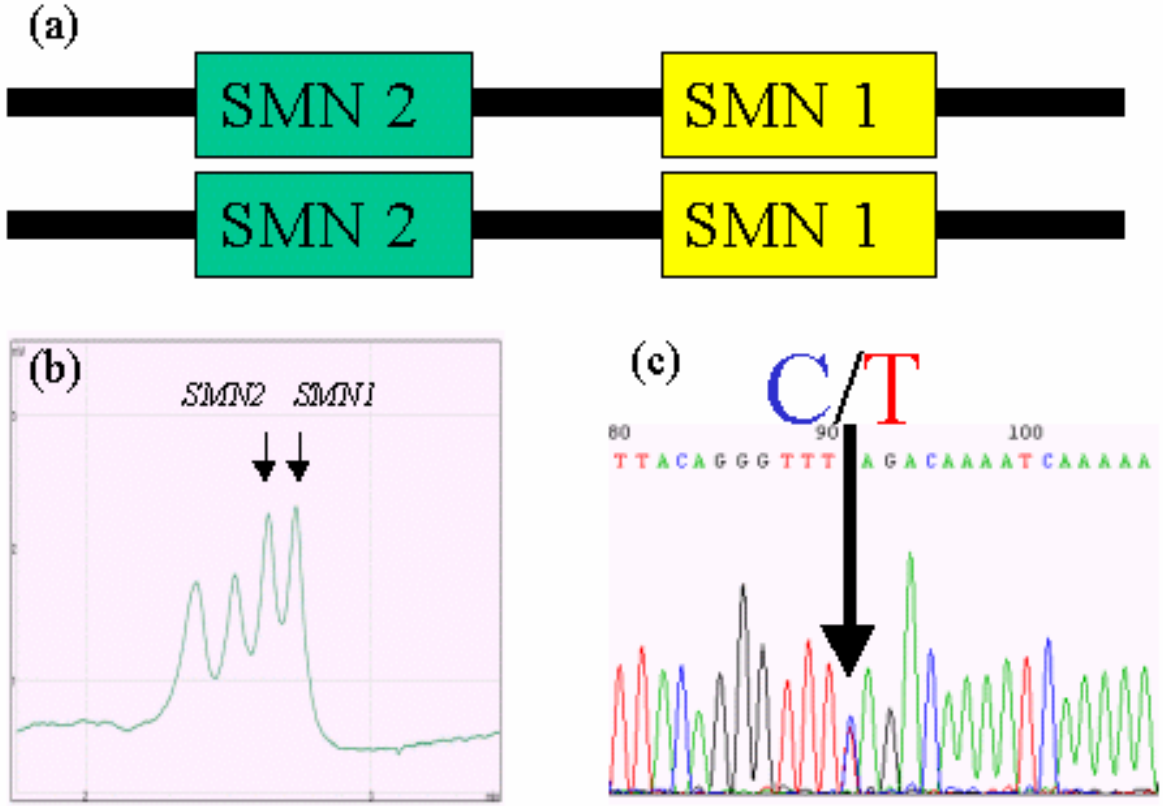


FIG.2 Chromatography and sequence analysis of an individual with equal dosage of SMN1/SMN2 genes:(a) genotype of wild type, (b) DHPLC, (c) sequences of exon 7

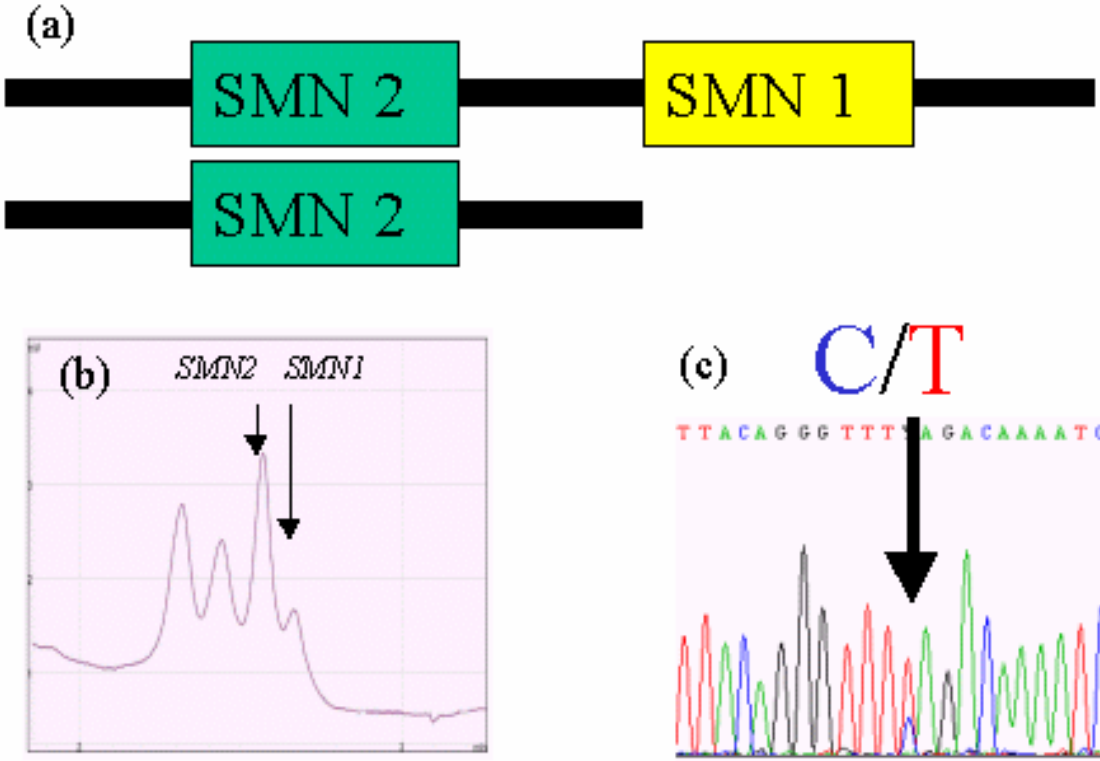
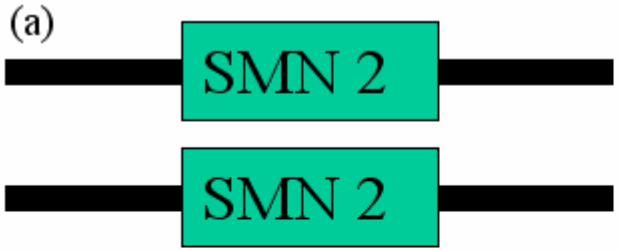
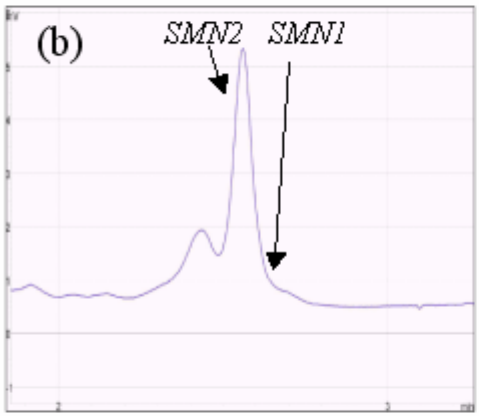


Fig.3 Chromatography and sequence analysis of an individual with a SMA carrier :(a) genotype of SMA carrier , (b) DHPLC, (c) sequences of exon 7





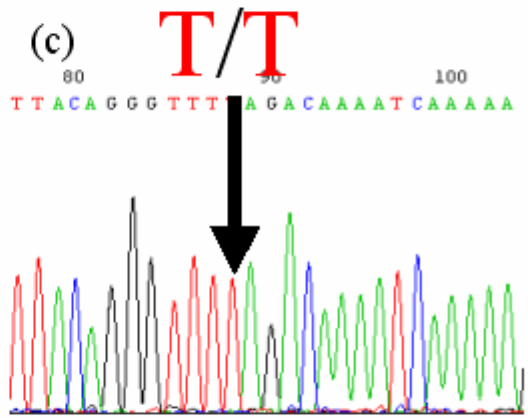


FIG.3 Chromatography and sequence analysis of an individual with an SMN2 gene only :(a) genotype of SMA affected, (b) DHPLC, (c) sequences of exon

Table. A total of 8 carriers of SMA and 7 patients with SMA lacking the *SMN1* gene as well as 72 control individuals from the general population and the family members of patients with SMA were analyzed

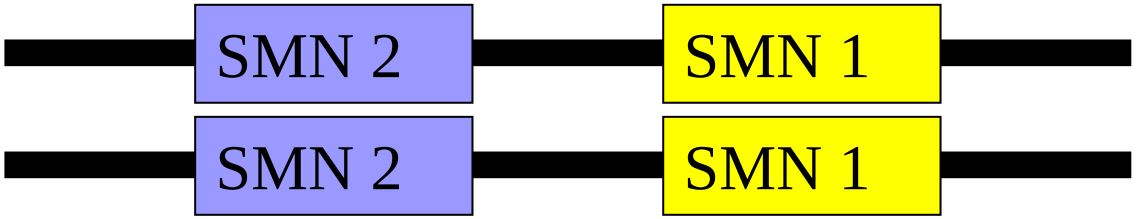
	The family member of patient with SMA	General population	Total
Normal	4	53	57
SMA carrier	6	2	8
SMA affected	7	0	7
Total	17	55	72

Conclusions

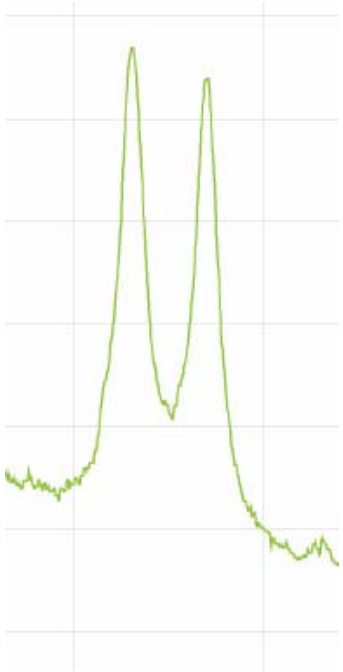
It is important to identify carriers of the *SMN1* absence for diagnostic purposes and genetic counseling.

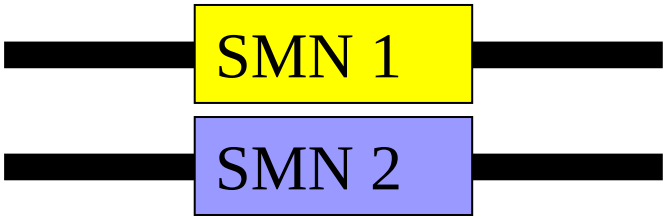
DHPLC is a fast and reliable tool for detection of carriers of SMA and SMA-affected patient with *SMN1* exon 7 deletion.

Misdiagnosis of SMA carrier

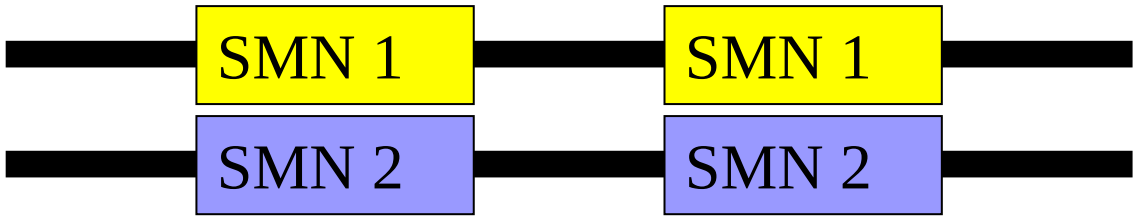


Wild Type





Carrier



Prenatal Diagnosis of Thalassemia

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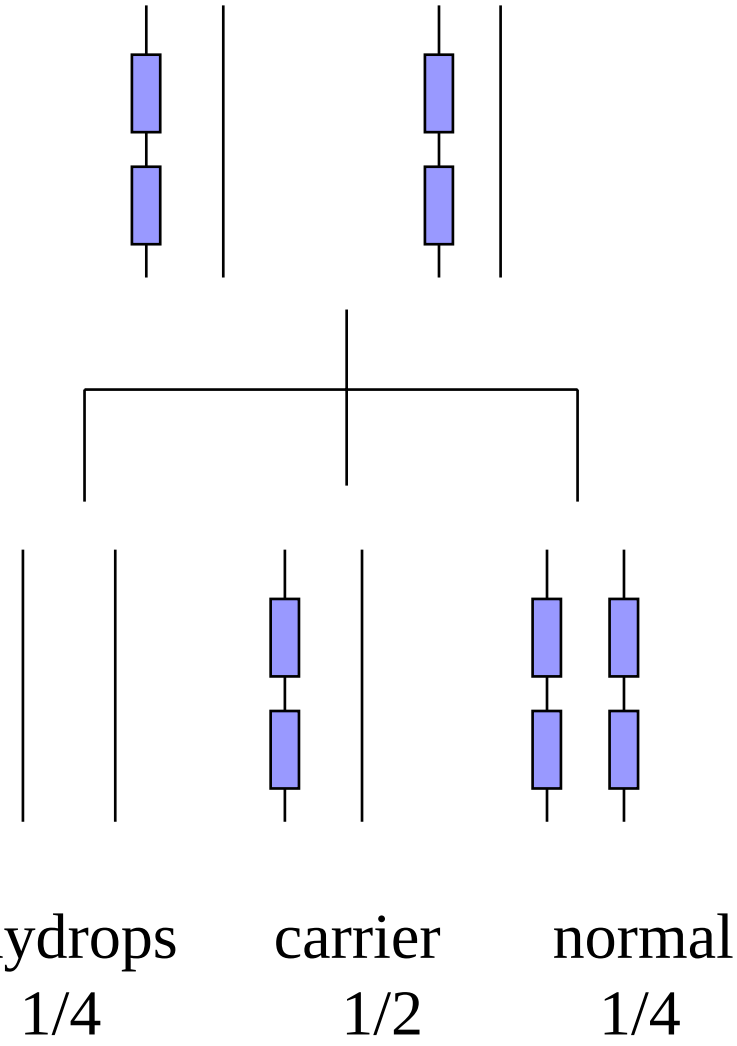
2006.01.18

α-thalassemia genotype

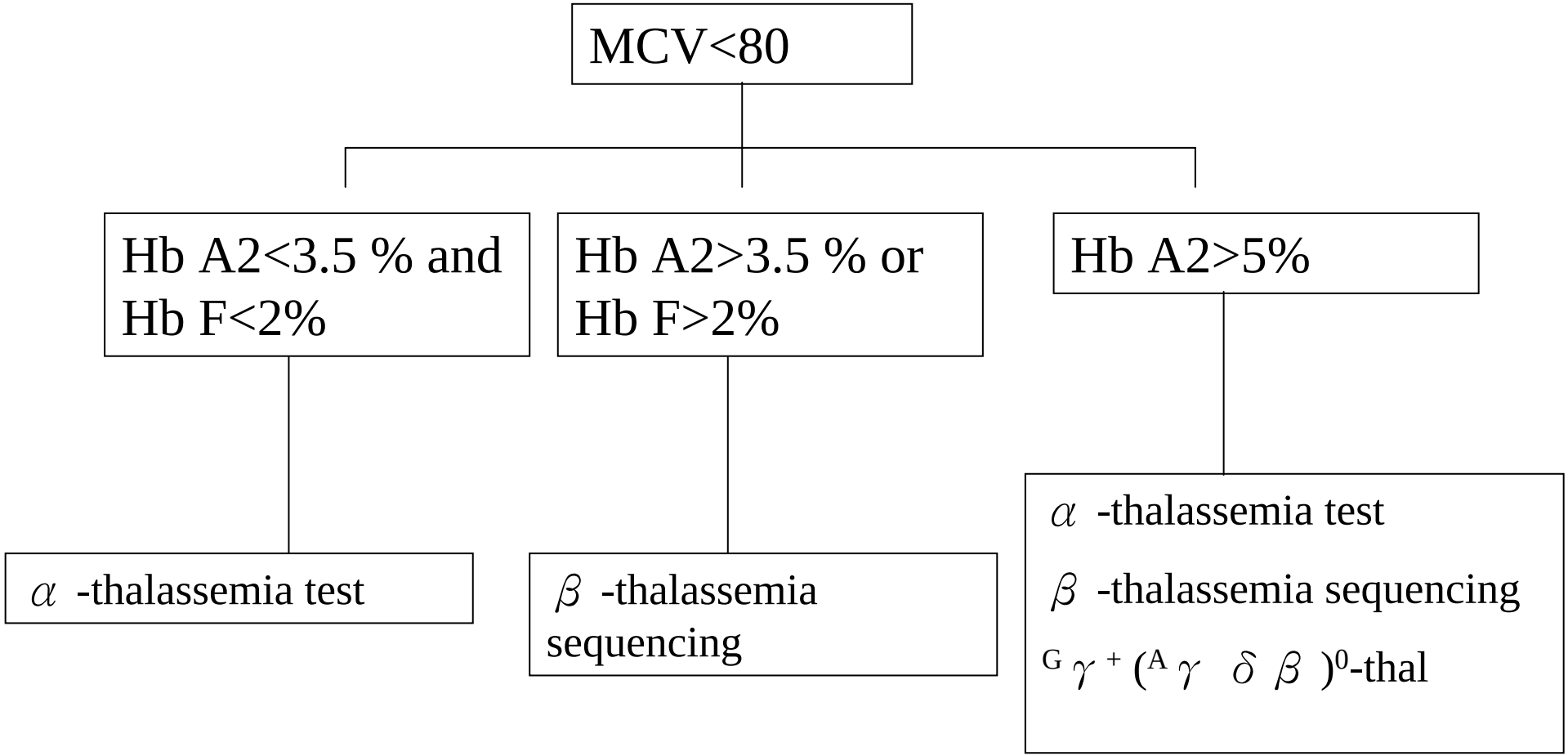
- Two gene deletion(--/αα)
  - SEA type (85%~96%)
  - Philippine type(1~3%)
  - others(<1%)
- One gene deletion (-α/αα)
  - -3.7 deletion(1~3%)
  - -4.2 deletion(1~2%)
- Point mutation(αα/αα^T)
  - Hb Constant Spring(1~3%)
  - Hb Quong Sze(<1%)
- Hb H disease (––/–α)



Screening for thalassemia

- CBC, Hb EP, Hb A_2 quantitation, Serum ferritin
- α-thalassemia
  - prevalence in Taiwan 3~7%
  - MCV < 80 fL , Hb A_2<3.5%
- β-thalassemia
  - prevalence in Taiwan 1~3%
  - MCV< 80 fL , Hb A_2 >3.5% or Hb F >2%

Thalassemia 檢驗流程

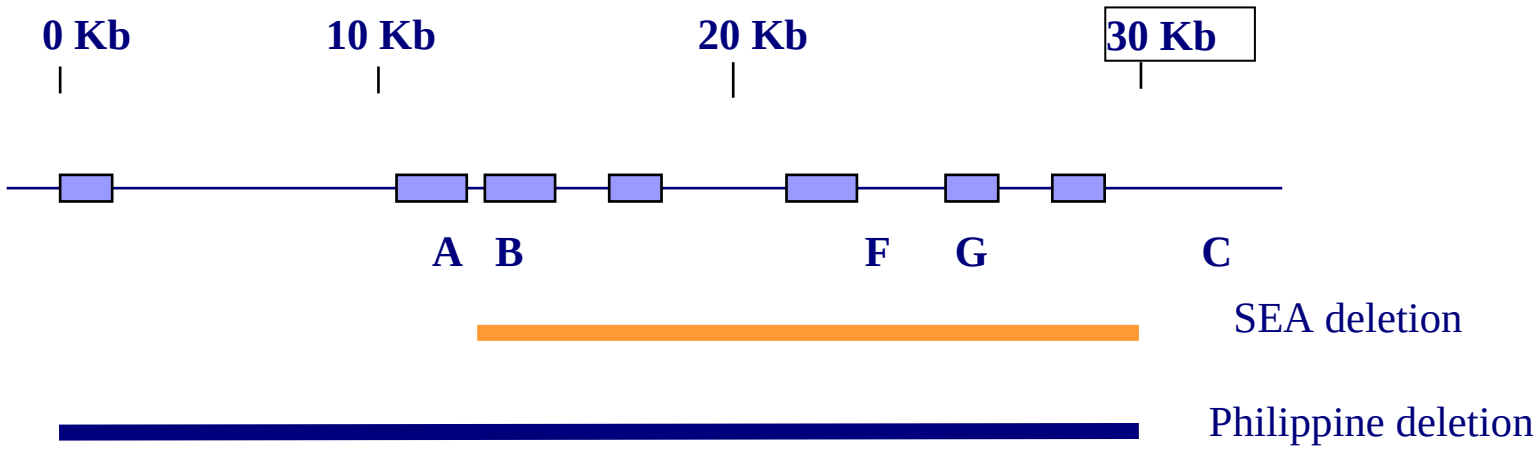


Prevalence of α thalassemia and β thalassemia in people in different areas of the Asia.

	α 1	α 2	β
Taiwan	4.0	1	1.1
Guangzhou	8.3	NA	3.4
South-East Asia	NA	>1-8	1-9
Thailand	10.0	10.0	5.0
Japan	NA	NA	0.1

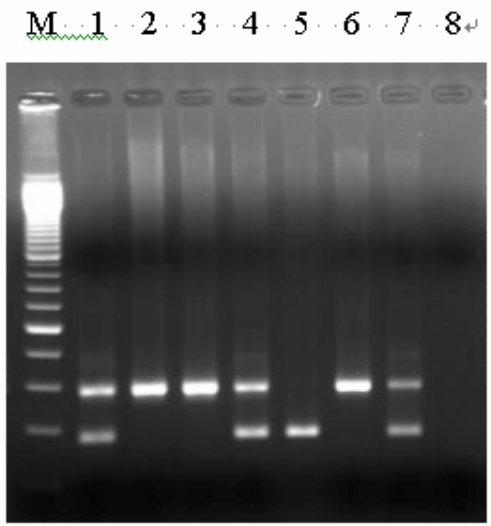
J Formos Med Assoc 1998; 97: 5-15 (Ko et al. 1998)

Prenatal diagnosis of α-thalassemia by PCR



Primers	A+C	A+B	F+G
偵測基因	SEA	Normal	Normal
Normal	-	+	+
Carrier	+	+	+
Hydrops	+	-	-

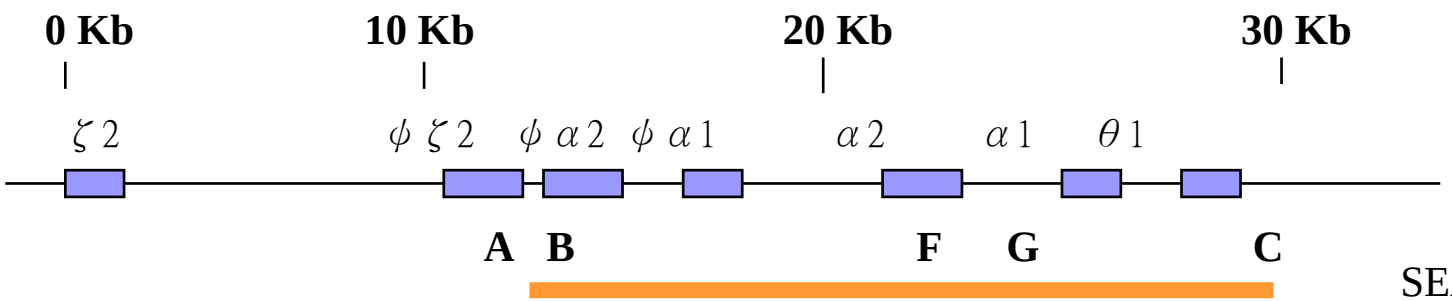
α-thalassemia-1 of Southeast Asia (SEA) type



M: 100 bp ladder
1: SEA carrier
2: wild type
3: wild type
4: SEA carrier
5:SEA homozygote
6: wild type
7: SEA carrier
8: H2O

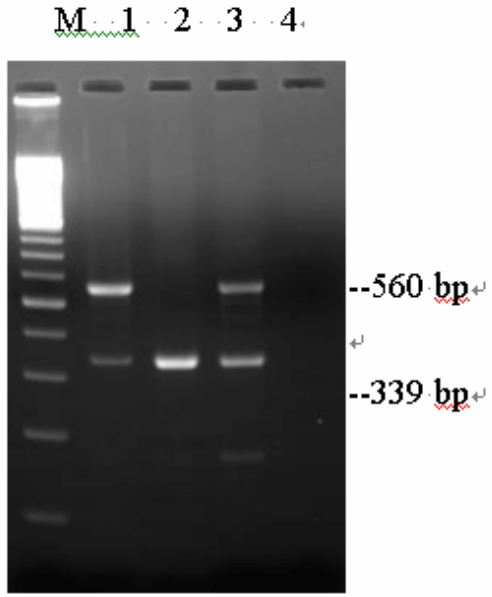
結果說明

1. SEA carrier 同時有 287 bp 與 194 bp 的 PCR 產物
2. wild type 僅出現 287 bp 的 PCR 產物
3. SEA homozygote 僅出現 194 bp 的 PCR 產物



SEA deletion

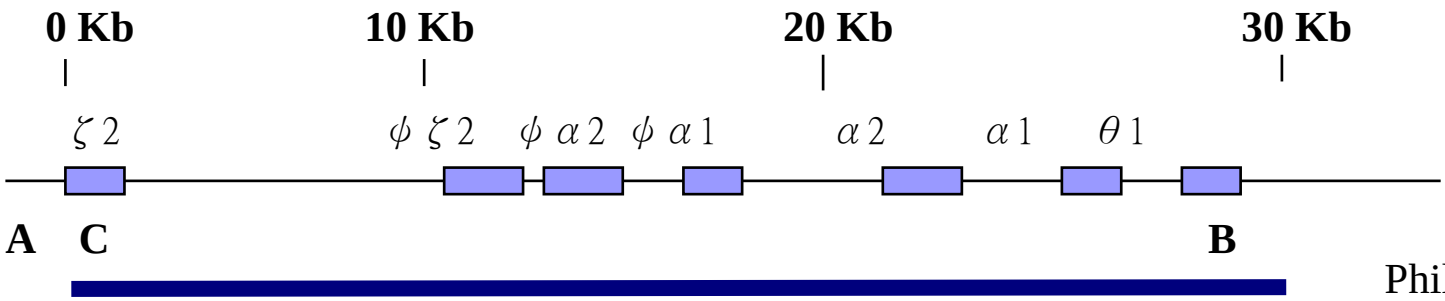
α-thalassemia-1 of Philippine deletion type



M: 100 bp ladder
1: Philippine carrier
2: Wild type
3: Philippine carrier
4: H2O

結果說明

1. Philippine carrier 同時有 560 bp 與 339 bp 的 PCR 產物
2. wild type 僅出現 339 bp 的 PCR 產物

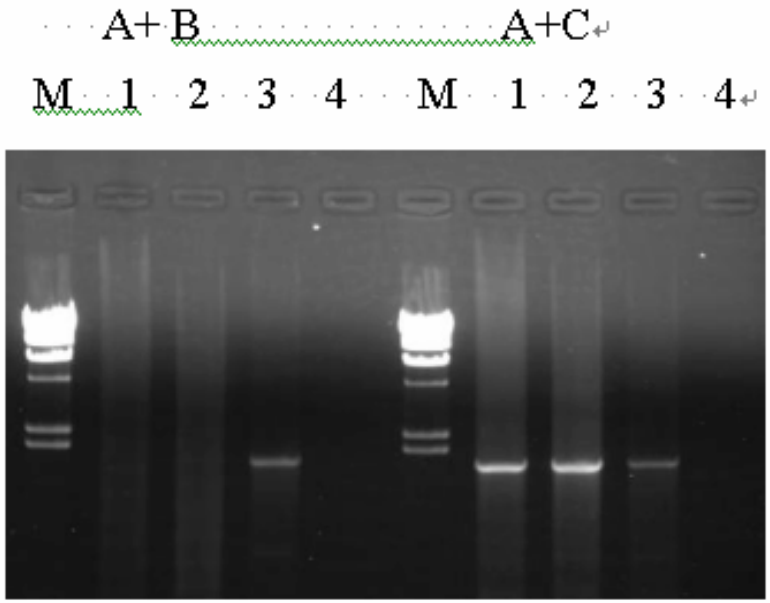


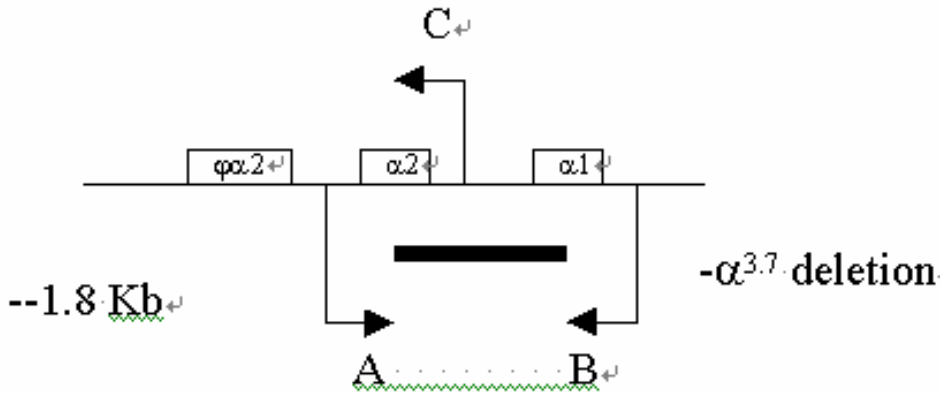
Philippine deletion

α-thalassemia-2 of -$\alpha^{3.7}$ deletion type

Primers A+B : for-$\alpha^{3.7}$ deletion ,1.8 kb

Primers A+C : for normal , 1.8 kb





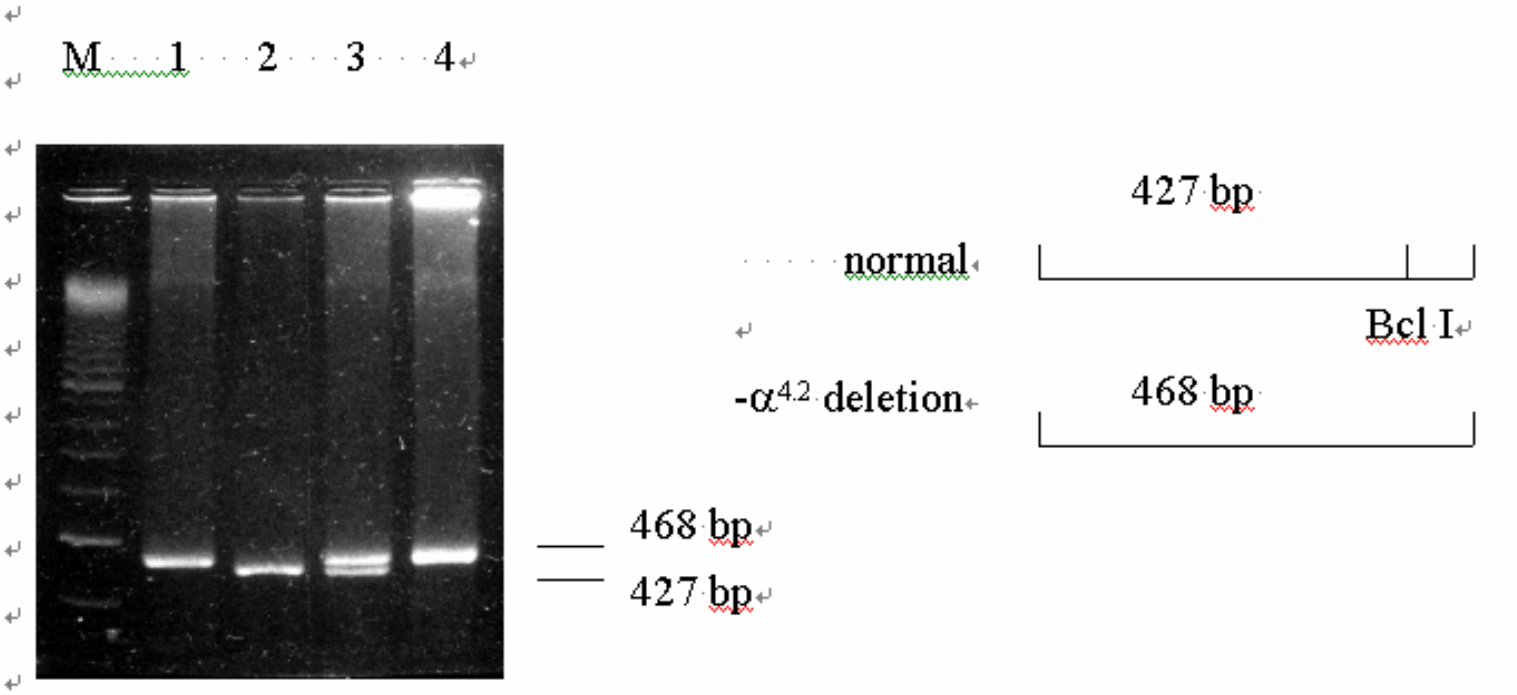
M : λ/Hind III marker

1 : Wild type

2 : Wild type

3 : -$\alpha^{3.7}$ deletion positive

α-thalassemia-2 of $-\alpha^{4.2}$ deletion type



M : 100 bp ladder

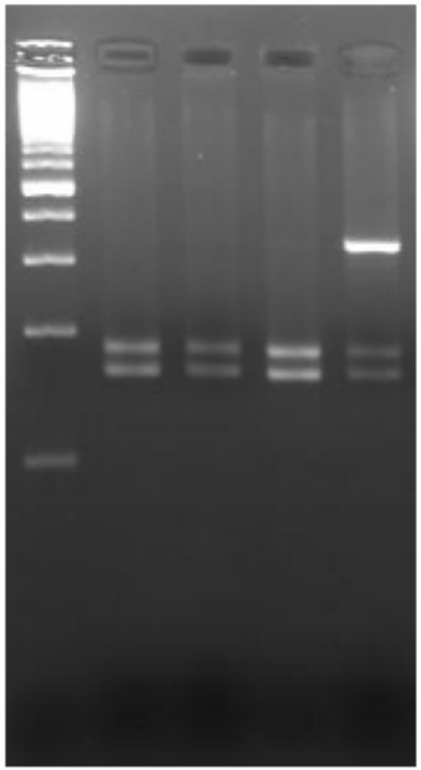
1 : normal undigested

2 : normal digested by Bcl I

3 : $-\alpha^{42}$ deletion carrier digested by Bcl I

4 : Hb H with $-\alpha^{4.2}$ deletion digested by Bcl I

Hb Constant Spring (Hb CS)

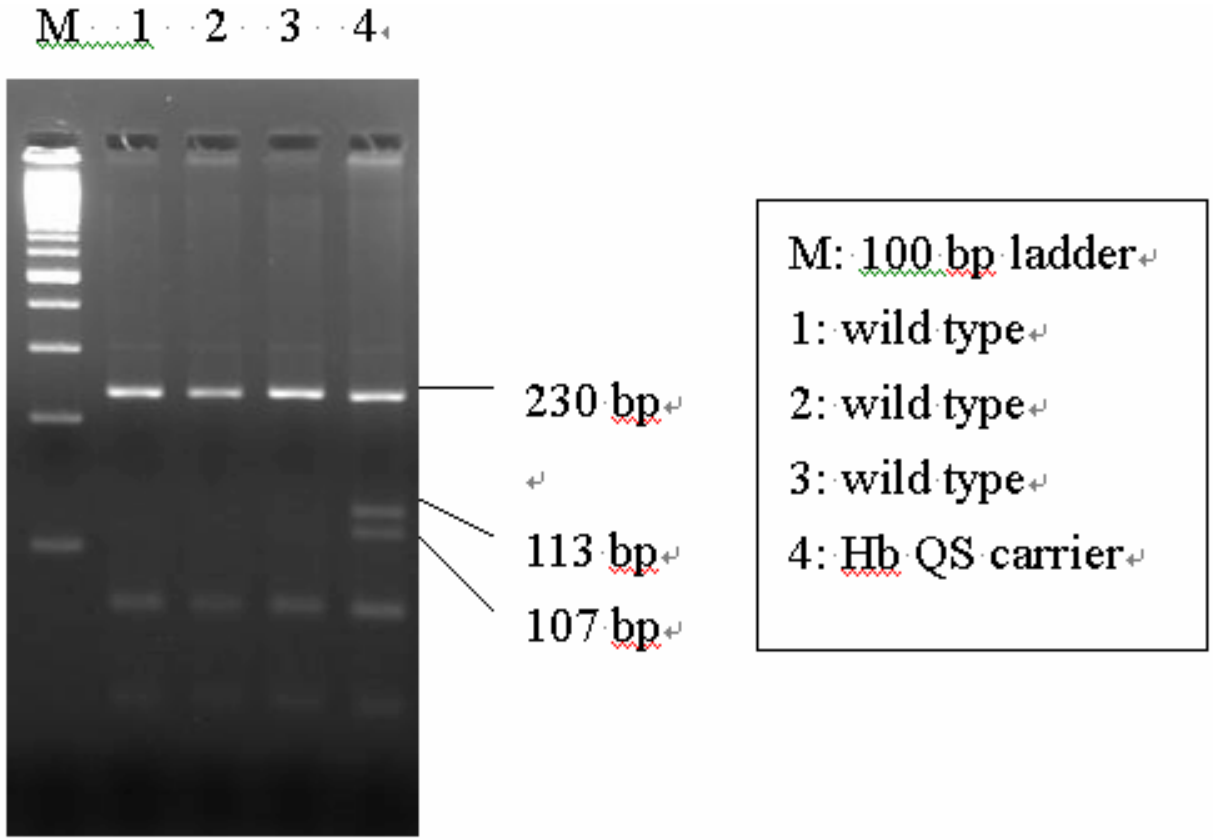


M: 100 bp ladder
1: wild type
2: wild type
3: wild type
4: Hb CS carrier

結果說明

1. Hb CS carrier 同時有 339 bp、182 bp 與 157 bp 的 PCR 產物
2. wild type 僅出現 182 bp 與 157 bp 的 PCR 產物

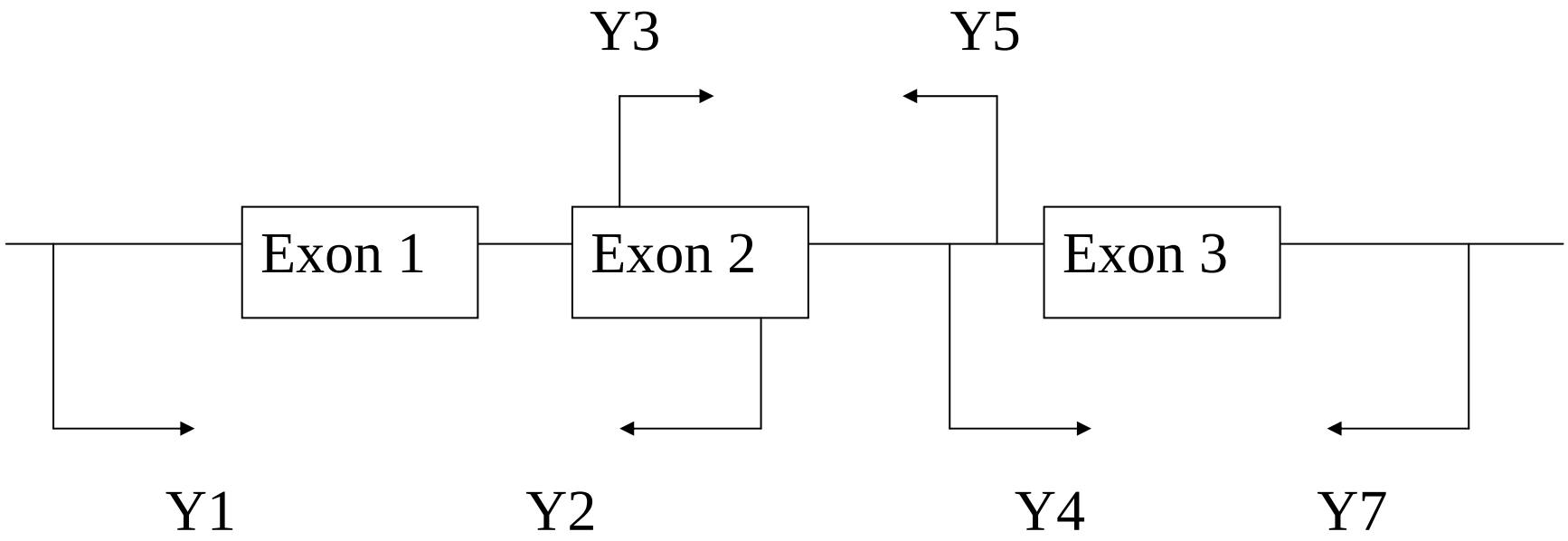
Hb Quong Sze(Hb QS)



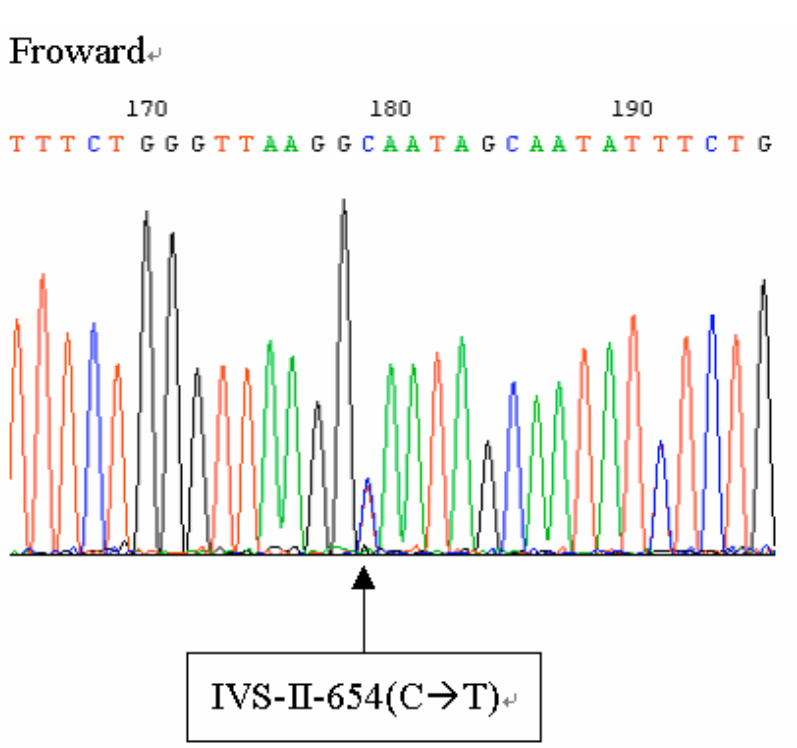
結果說明

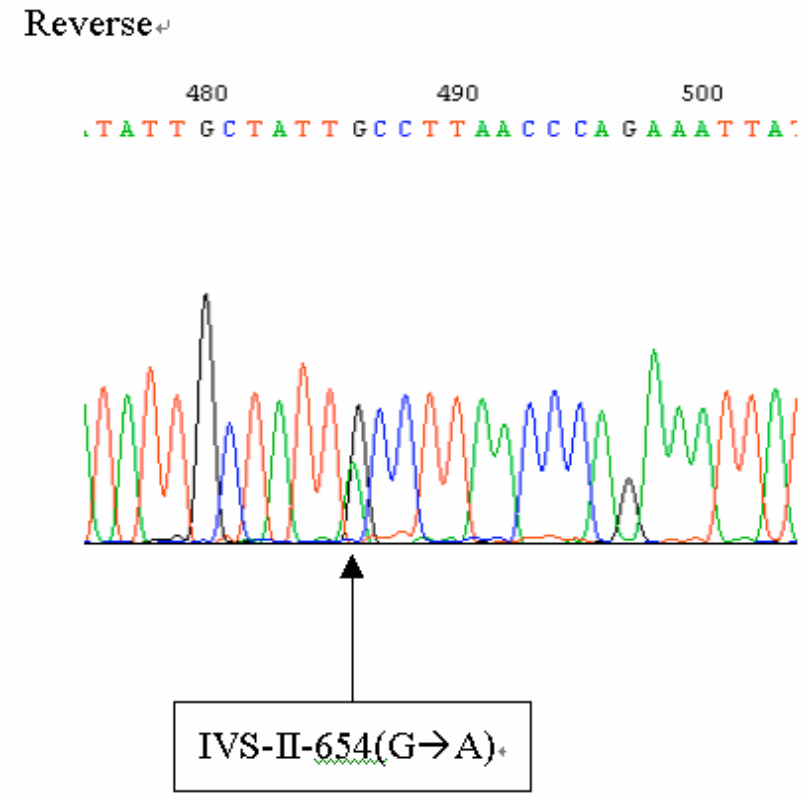
1. Hb QS carrier 同時有 230 bp、113 bp 與 107 bp 的 PCR 產物
2. wild type 僅出現 230 bp 的 PCR 產物

β-thalassemia 基因型分析

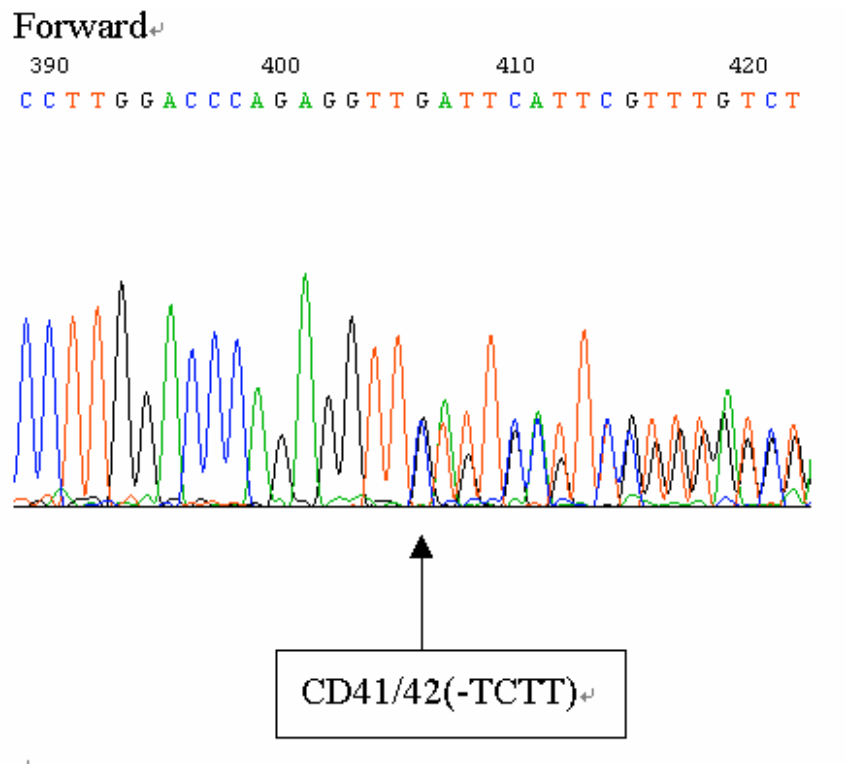


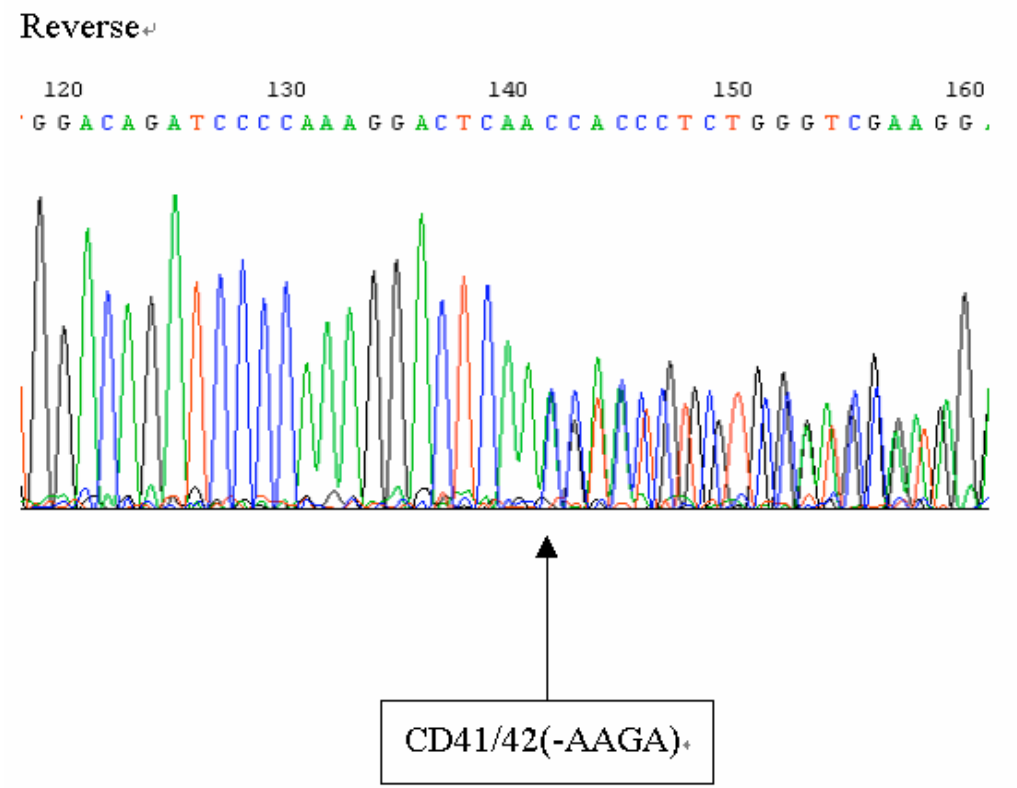
β-thalassemia of IVS-II-654(C→T)



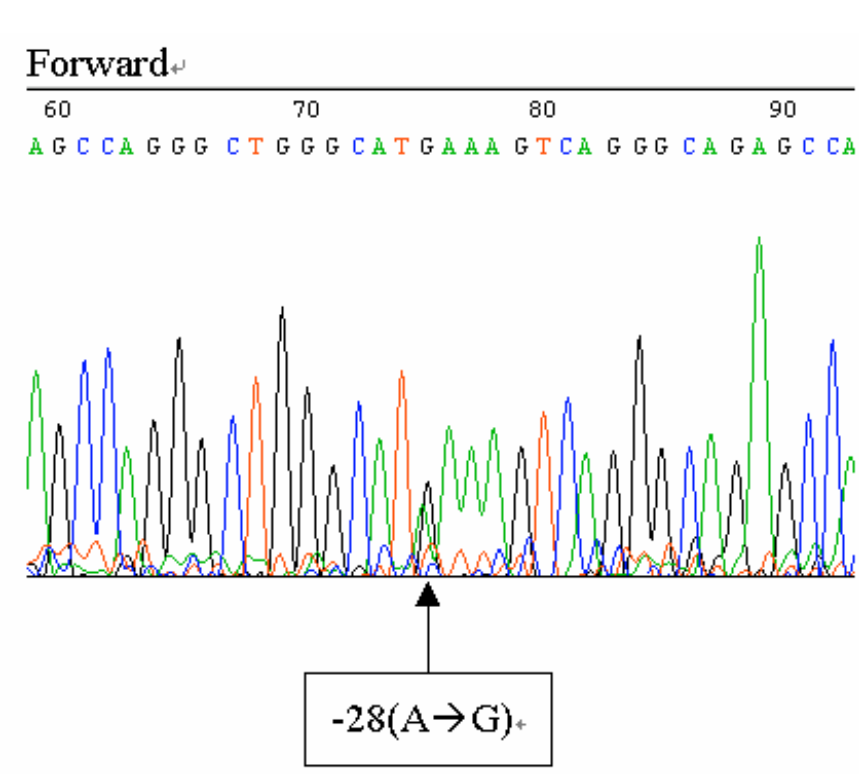


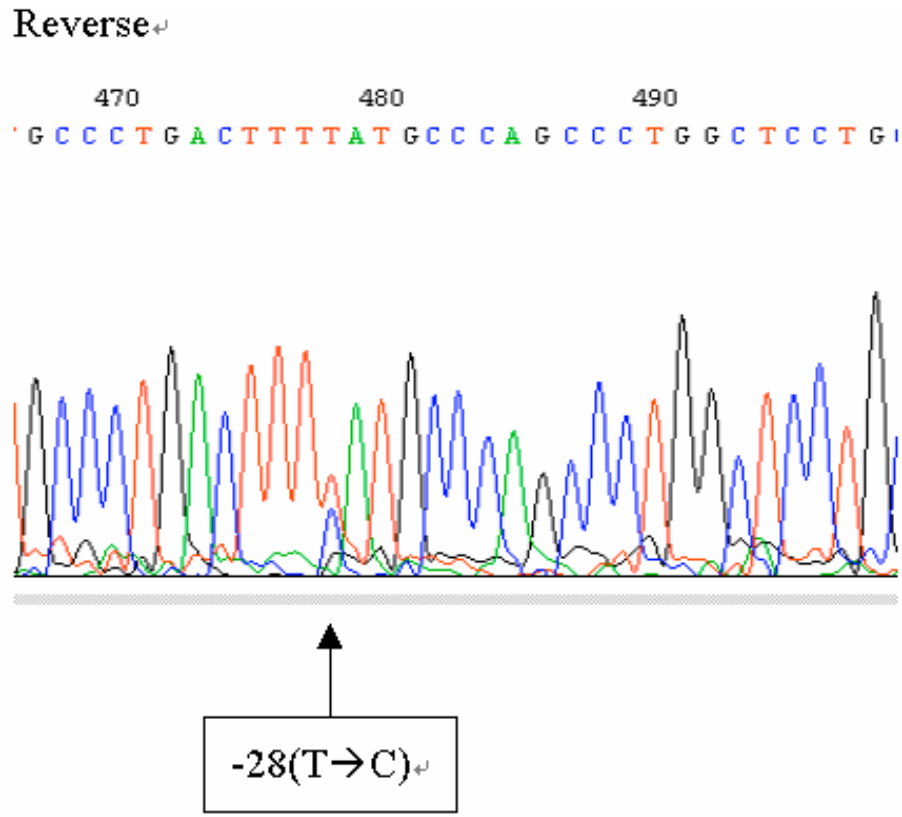
β-thalassemia of CD41/42(-TCTT)



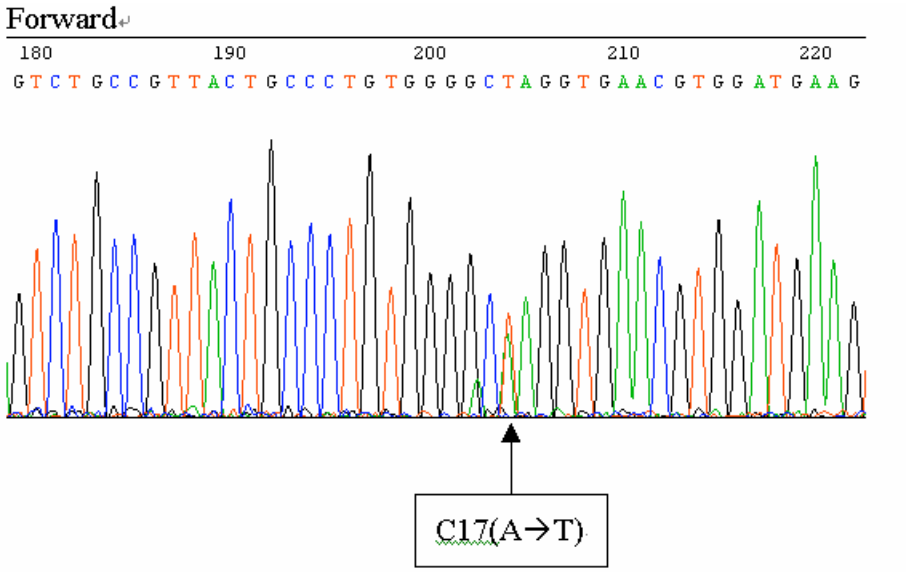


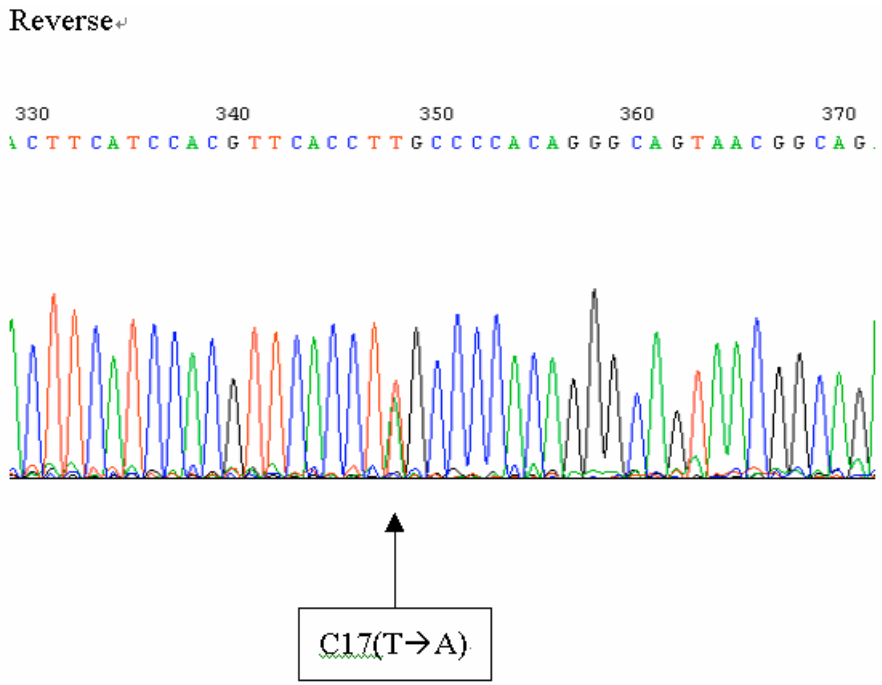
β-thalassemia of -28(A→G)



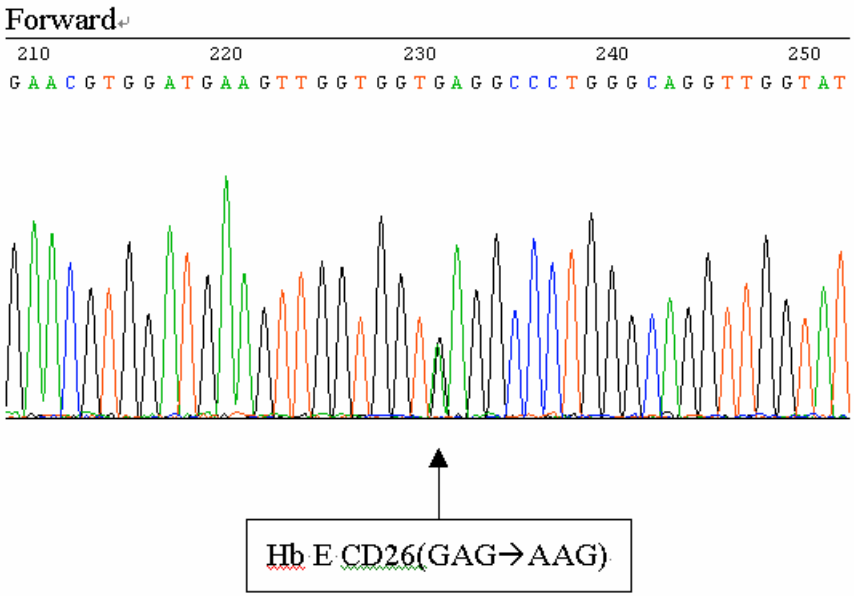


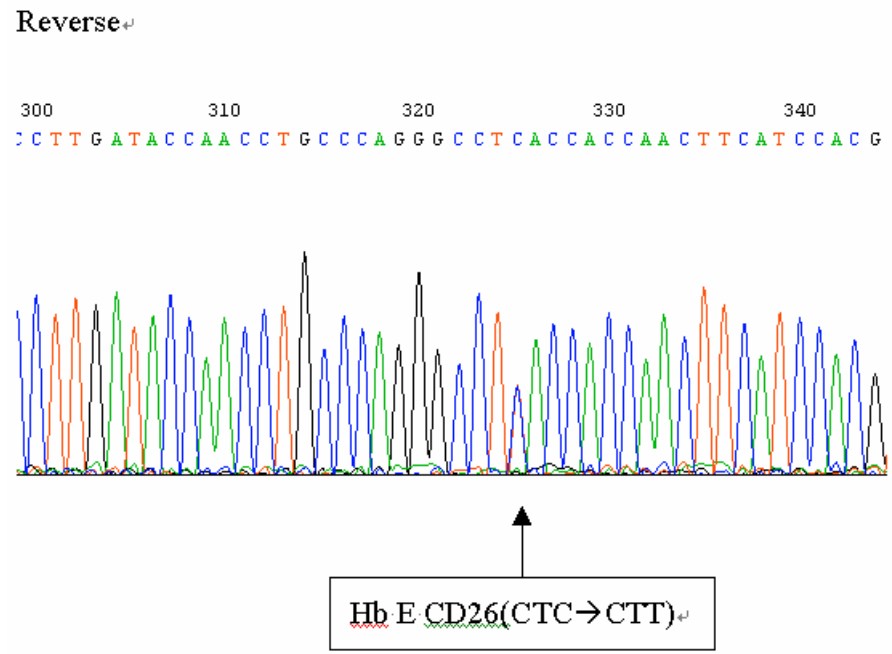
β-thalassemia of C17(A → T)



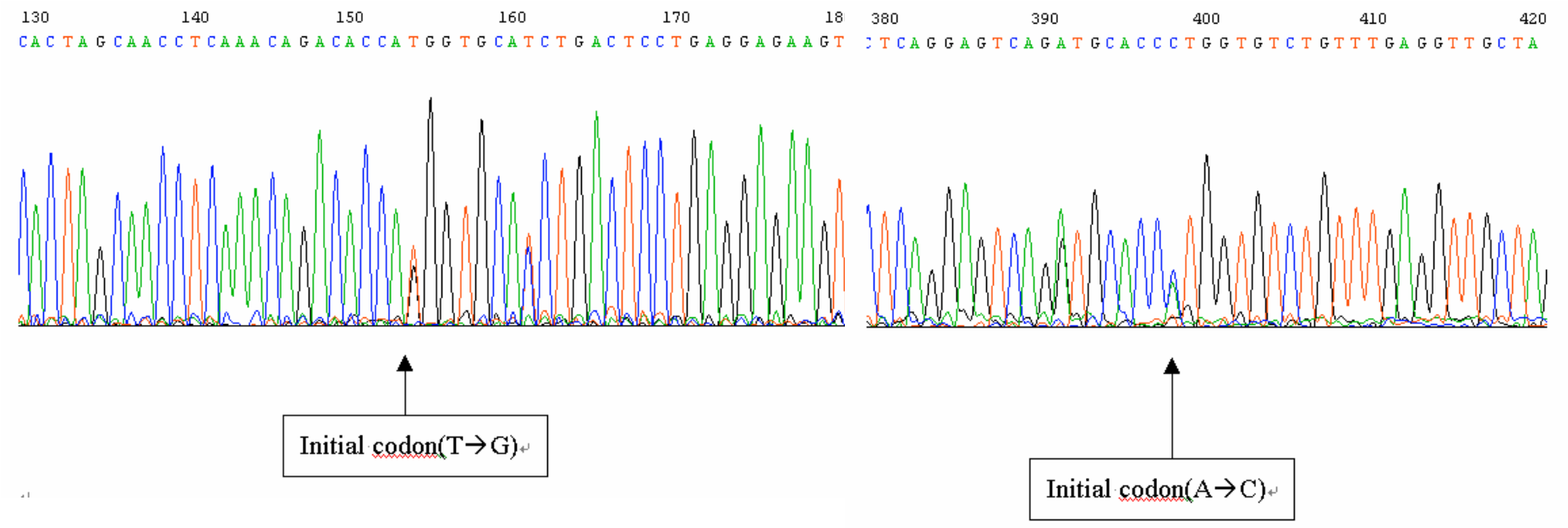


β-thalassemia of Hb E C26(GAG→AAG)

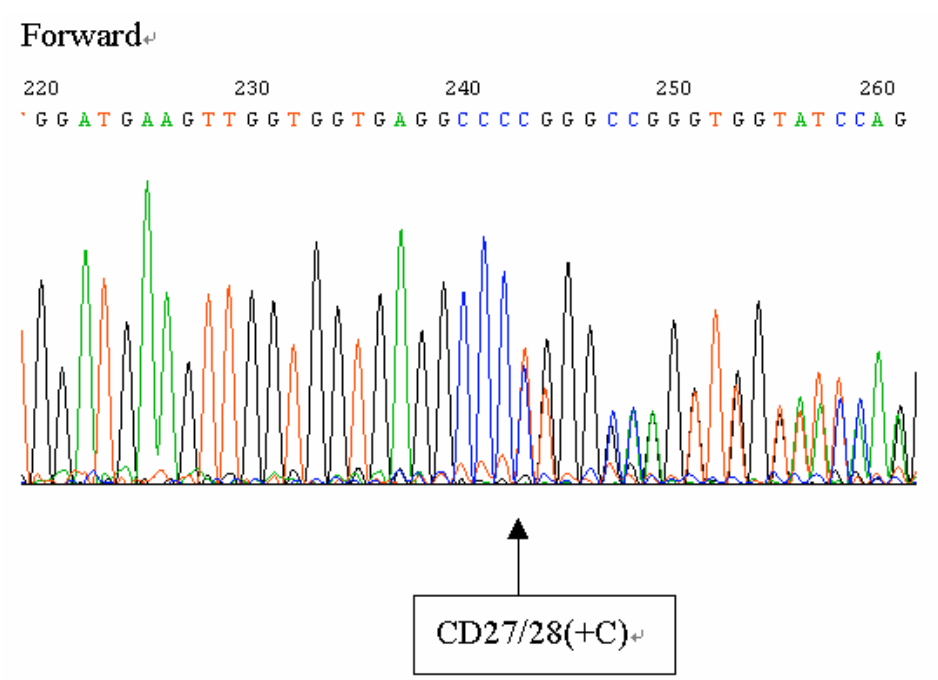


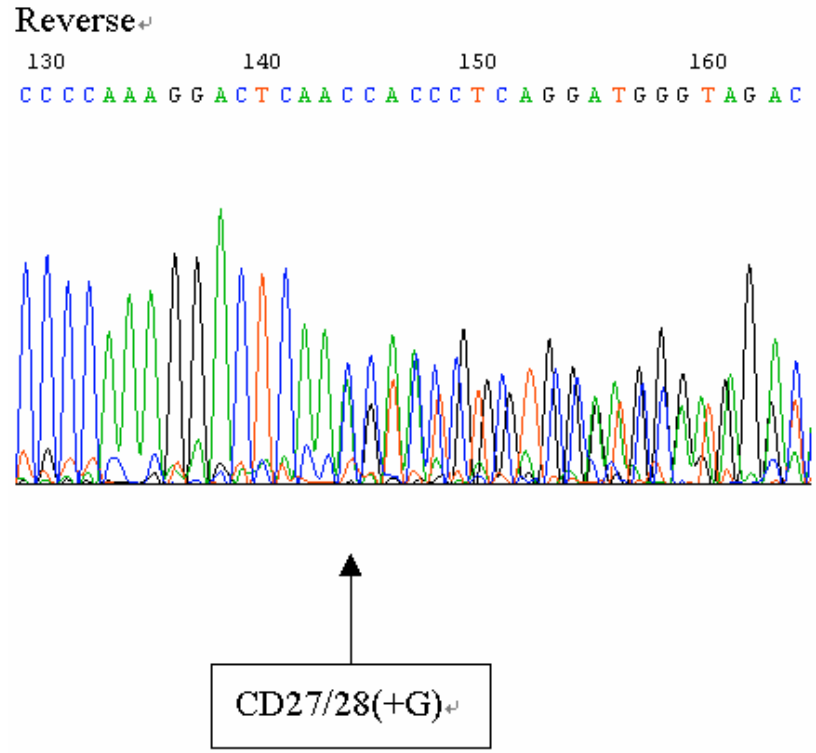


β-thalassemia of Initial codon(T→G)

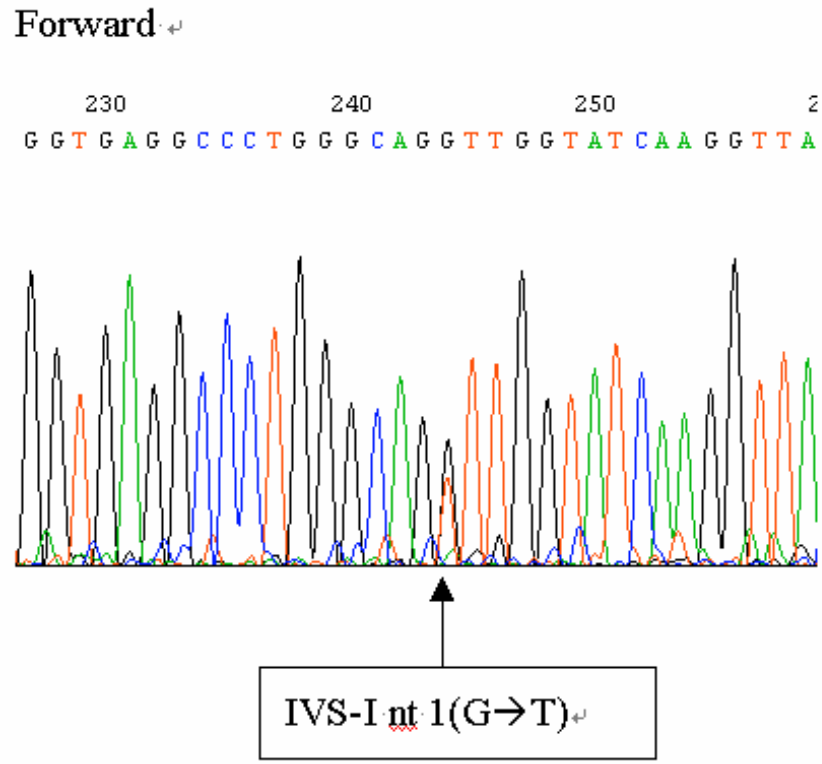


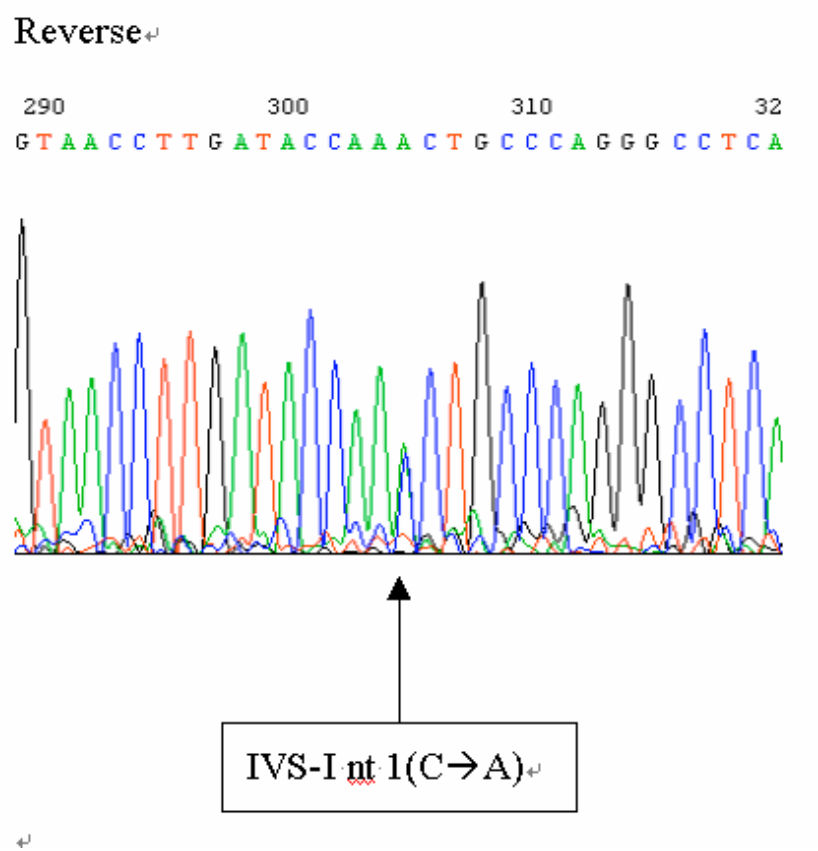
β-thalassemia of CD27/28(+C)





β-thalassemia of IVS-I nt 1(G→T)





α-thalassemia 六型基因型分析

SEA	Phi	3.7-del	42.-del	Hb CS	Hb QS	Other	Total
640	39	41	31	7	9	3	770
84%	5.1%	5.3%	4%	0.9%	1.1%	0.4%	100%

β-thalassemia 基因型分析

654	C41/42	-28	C17	Hb E	27/28	initial	IVS-I	Total
83	86	12	12	9	5	5	1	213
39%	40%	5.6%	5.6%	4.2%	2.3%	2.3%	0.5%	100%

6 common types of β thalassemia in Taiwan

	6 common types in Taiwan (in order)
Zhang et al. 1988	C41/42, nt654, C17, P28, IVS-I nt5, C71/72
Lin et al. 1991	nt654, C41/42, P28, C17, C27/28, P29
Ko et al. 1998	nt654, C41/42, P28, C17, C27/28, C26 (HbE)
Our lab. (87~93)	C41/42, nt654, P28, C17, C26 (HbE), C27/28 and Initial codon)

近三年內重大更動

<table>
<tr><th>時期</th><th>甲型 (HbA2< 3.5)</th><th>乙型 (HbA2> 3.5)</th></tr>
<tr><td>~92.8.26</td><td>先做SEA+PHI→若SEA (-), PHI(-)→加作其他四型</td><td rowspan="2">RFLP常見五型</td></tr>
<tr><td>92.8.26~</td><td rowspan="2">做常見六型</td></tr>
<tr><td>93.8.12~</td><td>Beta-sequencing</td></tr>
</table>

特殊案例-1: 林子文

Date	Hb	MCV	A2	F	Ferritin	Result
93.5.4*	8.5	84.8	2.5		1033.4	6 α-thal. (-)
93.5.14						C43 (+), nt654 (+)
94.3.28#	7.9	69	4.4	11.7	1131.3	P28 (+)

* After transfusion

\# After stem cell transplantation

特殊案例-2: 溫玉如

Date	Hb	MCV	A2	F	Ferritin	Result
93.8.13	7.3	61.5	5.6	3.1	107.8	6 α-thal. (-) 5 common β (-) Sequencing: Initial codon (ATG→AGG)

特殊案例-3: 陳韋杉

Date	Hb	MCV	A2	F	Ferritin	Result
93.8.20	8	79.8	2.2	7.8	904.9	6 α-thal. (-) 5 common β: nt654 (+) Sequencing: codon 67 (-TG): (+)

特殊案例-4: 許嘉玲

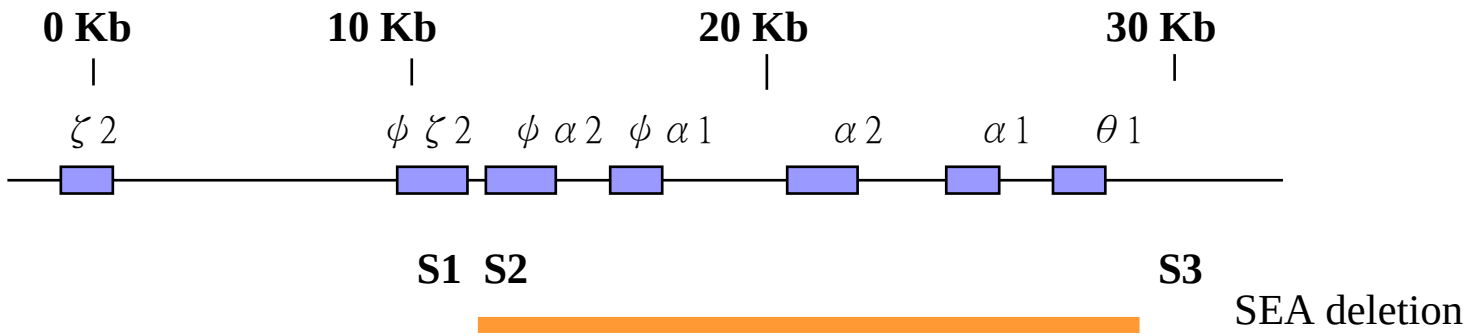
Date	Hb	MCV	A2	Ferritin	Result
93.3.7			3.0	31.6	Sequencing: Hb Siriraj (+) [β-gene codon 7 : GAG→AAG (Glu→Lys)]

財團法人長庚紀念醫院
林口醫學中心臨床病理科

地址：桃園縣龜山鄉復興街五號
電話：(03)3281200

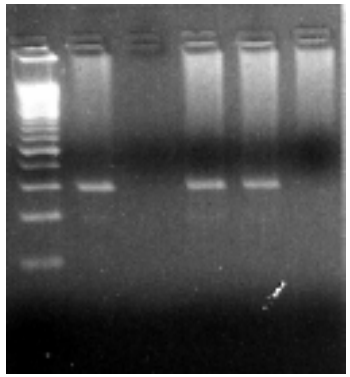
以及時定量PCR的方法可避免誤診同合子東南亞型甲型海洋性貧血

α-thalassemia-1 of SEA type Detection



Primer S1+S2(normal)

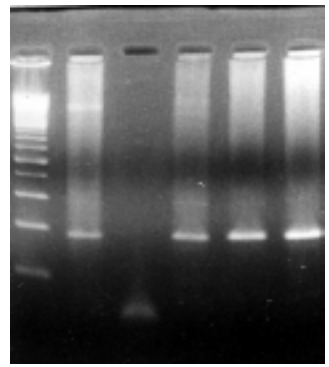
M 1 2 3 4 5



--287 bp

Primer S1+S3(SEA)

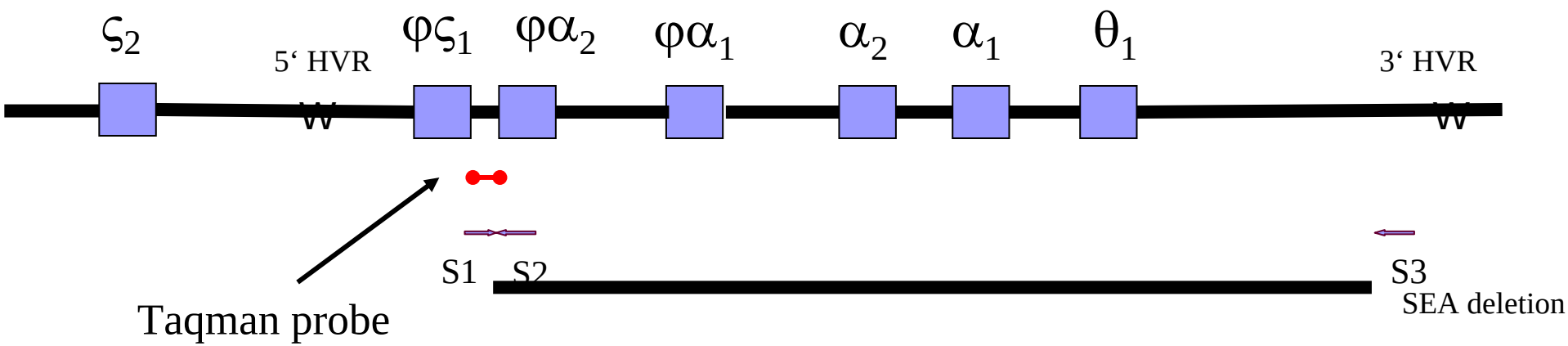
M 1 2 3 4 5



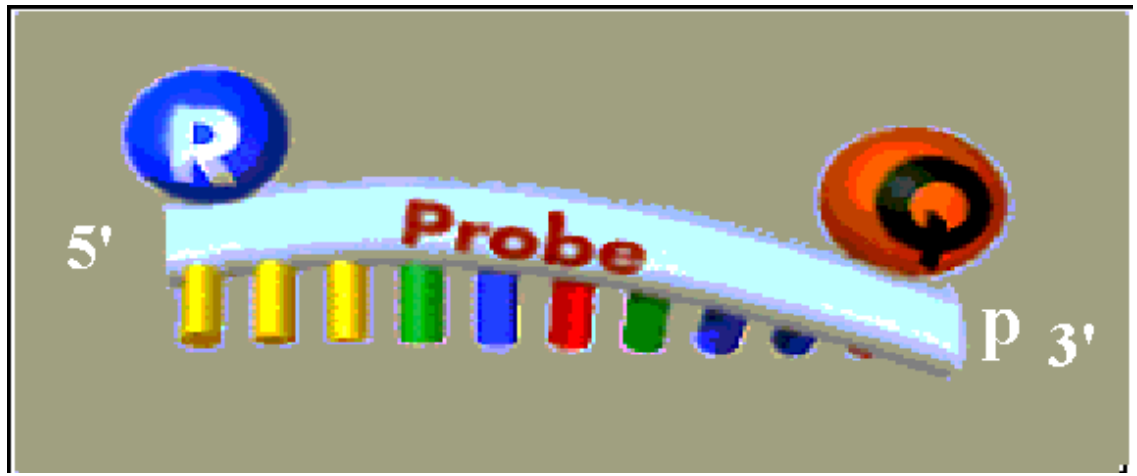
--194 bp

Hum Genet,1992, 88:254-248 . TM Ko et al

Real-Time PCR :
α-thalassemia-1 of SEA type Detection



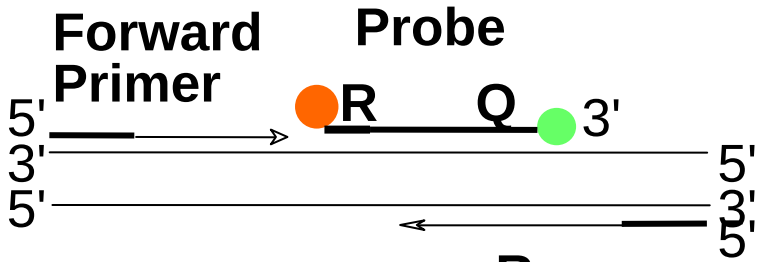
Probe Design



- 2 fluorescent dyes: R = Reporter Q= Quencher
- 3' end blocked (so it is not extended like a primer)
- High T_m (primers = 60 ˚C; probe = 70 ˚C)

Principle of Real-Time PCR

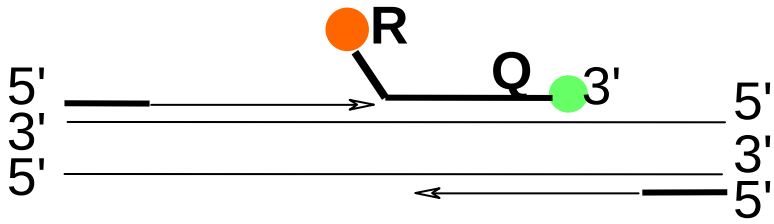
Polymerization



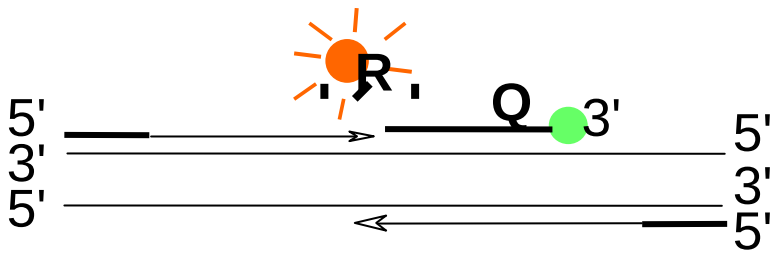
Q = Quencher

R = Reporter

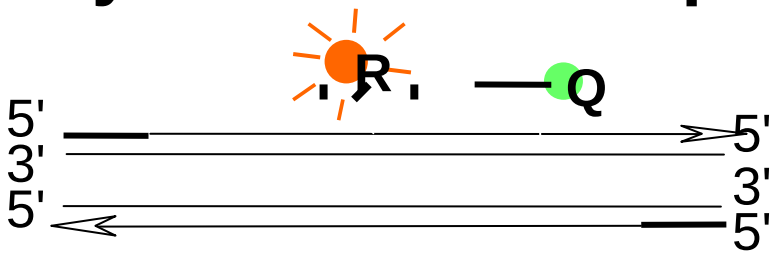
Strand displacement



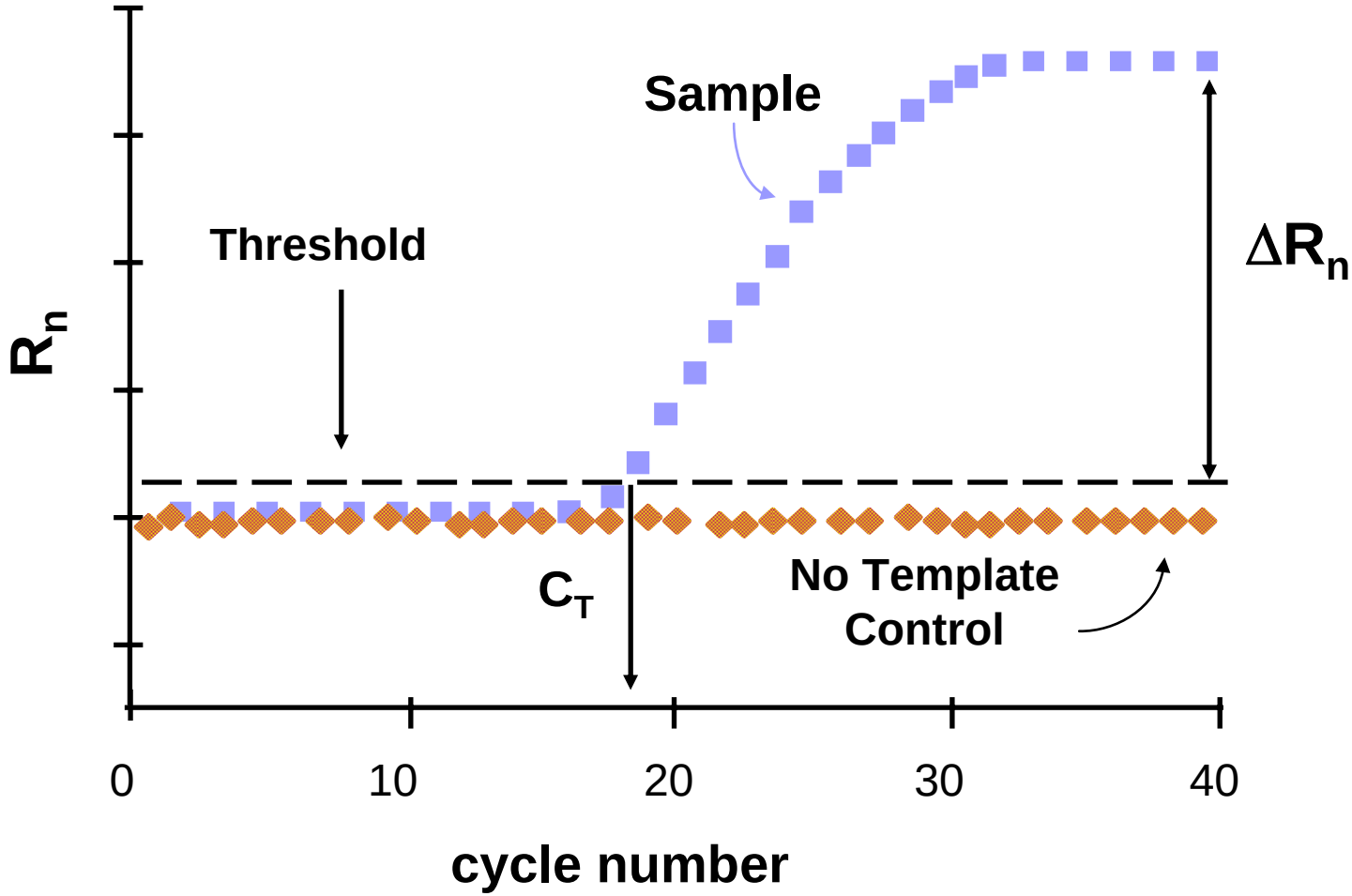
Cleavage



Polymerization completed



Theoretical PCR Amplification Curve



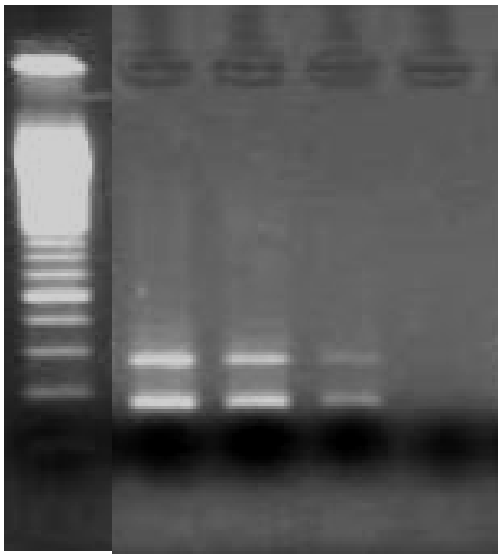
the C_T (threshold cycle)

—a measure of *when*—

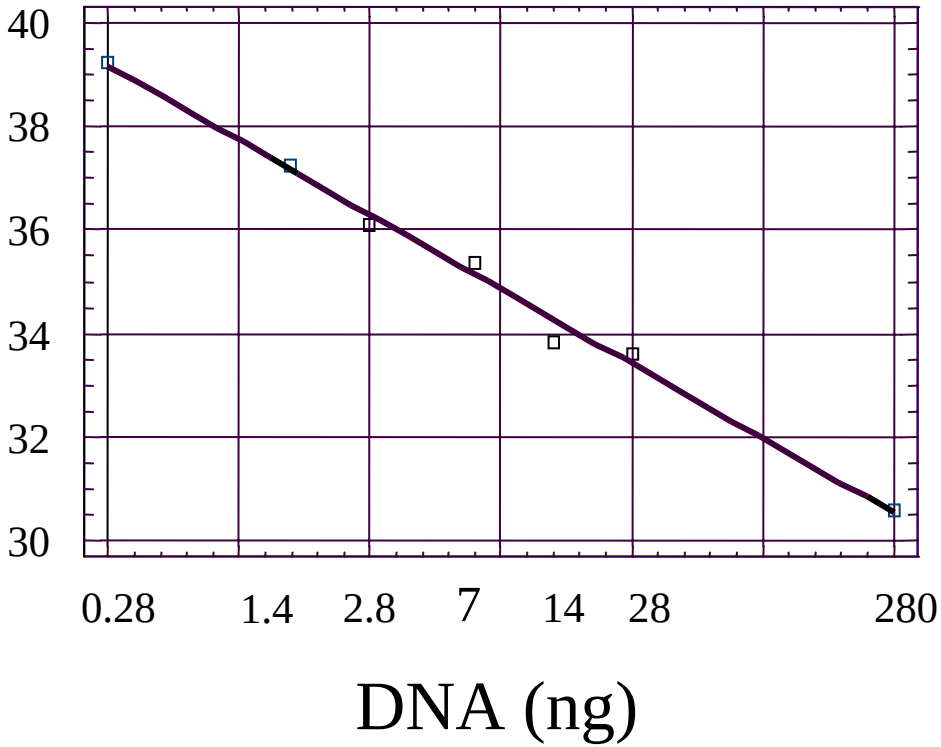
Sensitivity of traditional PCR and Real time PCR

y=30.54–2.87x
correlation coefficient= – 0.9965

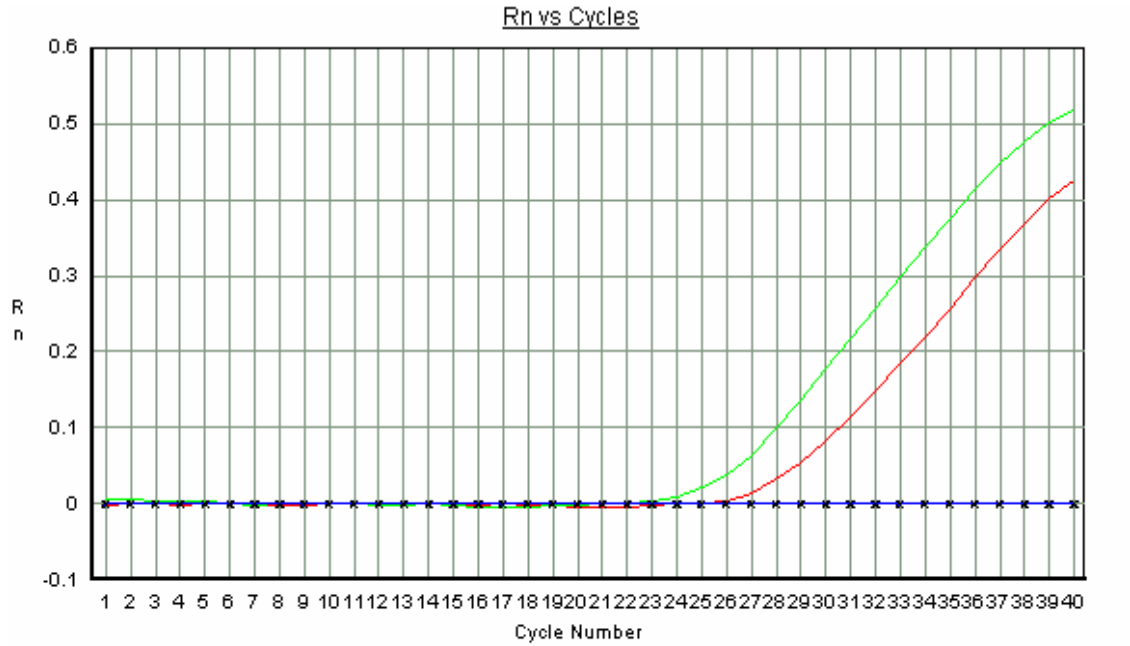
M 30 3 0.3 0.03 ng



C_T



Real-time Quantitative PCR Analysis for α-thalassemia-1 of Southeast Asian Type Deletion



← 1: S1+S3 for SEA

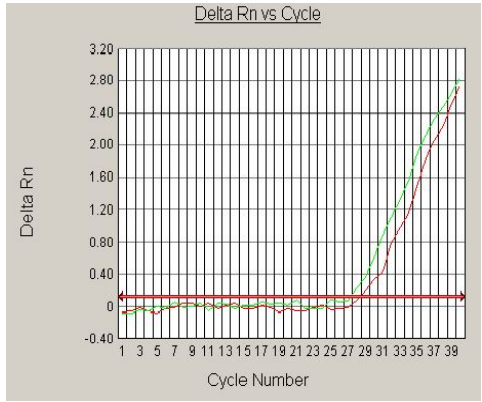
← 2 : S1+S2 for normal

← 3 : No template control

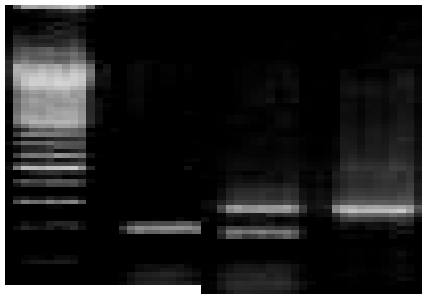
Real-time Quantitative PCR Analysis for α-thalassemia-1 of SEA Type Deletion

SEA homozygote

SEA Carrier



SEA Negative



Normal

SEA

Real-time Quantitative PCR Analysis for fetus samples

	S1+S2 for normal	S1+S3 for SEA
SEA carrier	27.67	26.41
SEA carrier	28.15	26.63
SEA homozygote	40	27.28
SEA homozygote	39.29	26.59
SEA homozygote	40	26.09
SEA negative	32.48	40
SEA negative	31.25	40

α-thalassemia-1 of SEA type Detection by traditional PCR

M : 100 bp marker

1 : cultured cells

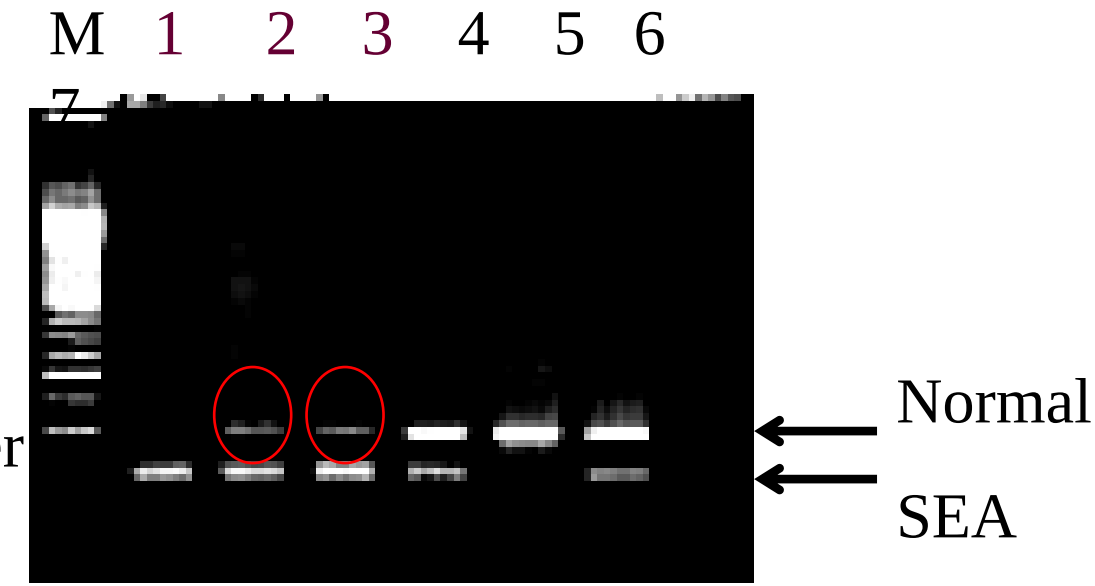
2 : placenta

3 : placenta

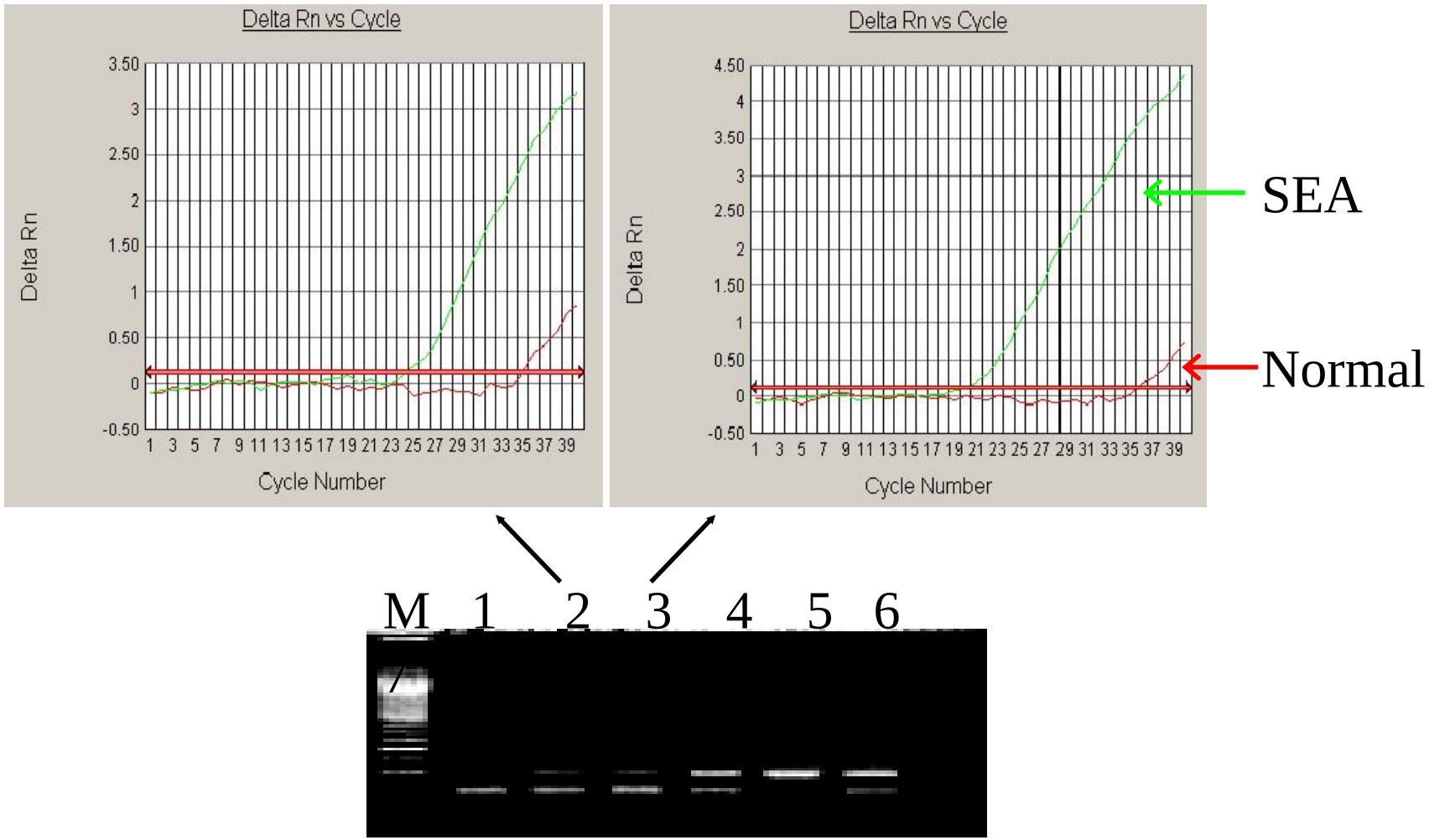
4 & 6 : SEA carrier

5 : SEA negative

7 : H_2O



α-thalassemia-1 of SEA type Detection by Real time PCR



如果SEA homozygote 的胎兒檢體被SEA carrier母體細胞污染

SEA homozygous DNA mixed with SEA carrier DNA

SEA homozygous sample mixed with SEA carrier sample

Homo : carrier	S1+S2 for normal	S1+S3 for SEA	ΔC_T
1 : 1/10	33.2	27.8	5.4
1 : 1/20	34.11	27.44	6.67
1 : 1/40	35.22	27.95	7.27
1 : 1/100	36.15	28.2	7.95
1 : 1/200	37.44	27.74	9.7
1 : 1/1000	39.56	27.74	11.82
1 : 1/10000	40	27.94	12.06
1 : 0	40	28.59	11.41